CHAPTER 4

Glycosidases from the *Moringa oleifera* seeds

4A. Purification and characterization of α-mannosidase (EC 3.2.1.24) from *Moringa oleifera* and the effect of glyco-conjugates on its activity.

4B. Purification, Biochemical characterization and localization studies of acidic α-galactosidase from *Moringa oleifera* seeds.

CHAPTER 4A

Purification and characterization of α-mannosidase (EC 3.2.1.24) from *Moringa oleifera* and the effect of glyco-conjugates on its activity

4A.1.0. Introduction

The enzyme α-mannosidases (E.C.3.2.1.24: α-D-mannosidase) are widely distributed in animals, plants and microorganisms [Kornfeld and Kornfeld, 1985]. They play a key role in biosynthesis and the turnover of *N*-linked glycoproteins which are involved in important biological activities in the cells [Hossain *et al.,* 2009]. To date, several α-mannosidases have been purified and characterized from different legumes [Li, 1967; Paus, 1977; Tulasi and Nadimpalli, 1997] and non legume sources [Ohtani and Misaki, 1983; Priya Sethu and Prabha 1997; Kishimoto *et al.,* 2001; Mahadevi *et al.,* 2002]. This enzyme is an abundant constituent of the plant hydrolytic system [Snaith, 1975]. α-mannosidases can be classified into two types: one is involved in the biosynthesis (or processing) of *N*-linked glycoproteins [Lerouge, 1998], the other in the degradation of *N*-glycans [Kimura *et al.,* 1999; Kishimoto *et al.,* 2001].The plant α-mannosidase that has been extensively studied is from the seeds of *Canavalia ensiformis* (jack bean) [Snaith, 1975; Howard *et al.,* 1998; Einhoff and Rudiger, 1998]. The Jack bean α-mannosidase was isolated by chromatography on concanavalin A, the lectin from the same plant, without involving its sugar binding site [Einhoff and Rudiger, 1986]. Jack bean α-mannosidase is a tetrameric protein, with two different subunits (66 kDa and 44 kDa), in which the larger subunit is glycosylated and it is zinc containing metalloprotein [Kimura *et al.,* 1999]. Furthermore, the formation of oligomeric structure with all the subunits present was found to be essential for the enzyme activity that is involved in the degradation of the *N*-glycoproteins. The α-mannosidases have been classified into two independently derived groups, Class I and Class II, based on the biochemical properties, substrate specificity, inhibitor profiles, catalytic mechanism and characteristic regions of conserved amino acid sequences and sequence alignments. In plant system this is based on the inhibitor profiles alone which characterize these groups [Daniel *et al.,* 1994; Moremen *et al.,* 1994; Eades *et al.,* 1998]. Jack Bean α-Mannosidases analogous to human lysosomal α-mannosidase are retaining glycosidases which are inhibited by Swainsonine a potent α-mannosidase II inhibitor [Howard *et al.,* 1997].

Plant α-mannosidases generally require metal ions for their activity, though zinc was found to be more common divalent metal ion required for their activities, other metal ions such as Co (II), Cd (II) were also found to enhance the enzyme activity [Nakajima *et al.,* 2003; Woo *et al.,* 2004]. The metal can be chelated using EDTA with loss of enzyme activity. The activity is regained by supplementing zinc externally [Snaith, 1975]. The involvement of the tryptophan residues at the active site was previously demonstrated in *Phaseolus vulgaris* (pinto beans) and Jack bean α-mannosidase [Paus, 1978; Burrows and Rastall, 1998]. It has been shown that the levels of the α-mannosidase increase during seed germination and fruit ripening suggesting its role in removing mannose residues from mannoglycans from the cell wall glycans [Kestwal *et al.,* 2007]. The enzyme has been used for the structural elucidation of glycoproteins and glycolipids. In particular, the α-mannosidase from jack bean whose properties have been extensively characterized has generally been employed in determining the glycan structure [Misaki *et al.,* 2003]. Furthermore, studies were carried out for designing potential glycosidase inhibitors derived from simple carbohydrates. It gives important information regarding the involvement of aromatic, imine and carbohydrate moieties of these inhibitors in effective inhibition [Kumar *et al.,* 2009].

Previously in our laboratory, we have purified and characterized α-mannosidase both from legume and non-legume sources (lablab beans and Triticale). This hydrolytic enzyme along with lectins is localized in protein bodies [Tulasi and Nadimpalli, 1997; Mahadevi *et al.,* 2002].

During the course of the initial studies on some biologically active proteins from *Moringa oleifera*, a multipurpose tropical plant belonging to the family of *Moringaceae* (order: brassicales), with special reference to lectins and glycosidases, it was found that seeds of *Moringa oleifera* contain significant levels of α-mannosidase activity. This plant grows quickly even on soils having relatively low humidity. It is known to be non-toxic to humans and animals. It is reported to contain coagulant protein, which has much practical significance [Gassenschmidt *et al.,* 1995].

In the present work we describe the purification and characterization of α-mannosidase from *Moringa oleifera* present in the seed kernel. Furthermore, we also obtained information about the transition state analogues inhibition studies to this enzyme which will help in homology modeling.

4A.2.0 Materials and methods

4A.2.1. Materials

Moringa oleifera seeds (PKM_1) variety was purchased from the local market. *p*-Nitrophenyl-α-D-mannopyranoside, other *p*-nitrophenyl glycosides, α-methyl-D-mannopyranoside, Sephacryl S-200 HR and phenyl-Sepharose CL-6B was obtained from Fluka (Sigma-Aldrich), Con A Sepharose 4B gel supplied by Amersham (GE Healthcare), Uppsala, Sweden, DE-52 (DiEthyl cellulose) was obtained from Whattman, Ready to use standard protein molecular weight marker mixture for SDS-PAGE was obtained from Fermentas. Synthetic glyco-conjugates used in these studies were kindly provided by C. P. Rao, Department of Chemistry, IIT Bombay. All other chemicals and reagents were of analytical grade and procured from reputed firms.

4A.2.2. Enzyme assays

Under the standard test conditions, α-mannosidase activity was measured by the release of *p*-nitrophenol from the chromogenic substrate *p*-nitrophenyl-α-D-mannopyranoside (*p*NP-α-Man) 5 mM, stock. An assay mixture (500 µL) consisting of a 100 mM acetate buffer pH 5.0, 100 µL of a 5 mM *p*-nitrophenyl-α-D-mannopyranoside (1 mM final concentration) and the enzyme solution, was incubated at 37°C for 30 min. The control contained all reactants except the enzyme. Determination of other *p*-nitrophenylglycosidase activities was carried out under the same experimental conditions. The reaction was stopped after 30 min of incubation by addition of 1 M Na_2CO_3 buffer pH 10.0 to the reaction mixture. Liberated *p*-nitrophenol was measured spectrophotometerically at 405 nm.

The activity of the enzyme (units/ml/min) was calculated according to the formula given below:

$$\text{Activity (units/mL/min)} = \frac{\text{Absorbance at 405nm}}{\text{Time of incubation x 18.5 x Volume of enzyme}}$$

Where 18.5 is the molar extinction coefficient of *p*-nitrophenol

Definition of enzyme unit: One unit of enzyme activity was defined as amount of enzyme hydrolyzing 1 µmoL of substrate per min. The specific activity of the enzyme was expressed as units per mg protein. (IU/mg). All assays were performed in triplicate and results were recorded as the mean of these experiments.

4A.2.3. Extraction of *M.oleifera* α-mannosidase from seeds

Unless otherwise stated, all the purification steps were carried out at 4°C. Protein was monitored in the column fractions of various chromatographic steps by measuring the A_{280} in spectrophotometer. Enzyme activities were also monitored as described above.

Seeds of *M.oleifera* (PKM_1) were deshelled just before the extraction and the kernel was ground using a kitchen blender. Seed powder was defatted using chilled acetone, after removal of acetone by centrifugation the solids were air dried at room temperature. Total protein from 50 g of seed powder was extracted overnight with ten volumes of 25 mM Tris-HCl pH 7.4. After extraction the homogenate was centrifuged at 12,000 rpm for 30 min. The pellet was discarded and the supernatant which was considered as the crude extract of the enzyme preparation was subjected to 0-40% ammonium sulphate precipitation. After pelleting at 12,000 rpm for 30 min, the precipitated protein was collected and the supernatant was again saturated to 40-80% ammonium sulphate. The fraction of 40-80% precipitate which is found to be rich in α-mannosidase activity was dissolved in 25 mM Tris-HCl pH 7.4 and dialysed against same buffer.

4A.2.4. Anion exchange chromatography on DE-52

The 40-80% ammonium sulfate precipitated enzyme sample after dialysis was then loaded on to the DE-52 (4 x 9 cm), that has been previously equilibrated with 25 mM Tris-HCl pH 7.4. The unbound proteins were removed from the column by washing with five column volumes of the same equilibrating buffer. The absorbed proteins were then eluted using stepwise gradient of NaCl from 0.05–3 M NaCl in the same buffer. Fractions (1 mL each) were collected at a flow rate of 60 mL/h and assayed for the enzyme activity. The active fractions containing α-mannosidase were pooled and concentrated by Amicon concentrator.

4A.2.5. Hydrophobic interaction chromatography using phenyl-Sepharose CL-6B

The pooled and concentrated enzyme rich fractions from the previous step was saturated to a final concentration of 1.0 M with ammonium sulfate and applied on a phenyl-Sepharose CL-6B column (1.5 x 5 cm) previously equilibrated with 25 mM Tris-HCl pH 7.4 containing 1 M $(NH_4)_2SO_4$. The column was washed with equilibration buffer and the bound proteins were then eluted with 25 mM Tris-HCl pH 7.4. Fractions of 1 mL were collected at a flow rate of 1mL/min and active fractions were pooled together. The pooled fractions were concentrated and were dialysed in 25 mM Tris-HCl pH 7.4 buffer.

4A.2.6. Affinity purification on Con-A Sepharose 4B column

The concentrated fractions from phenyl-Sepharose were applied on to a Con A-Sepharose 4B column (5 ml) previously equilibrated with 25 mM Tris-HCl pH 7.4 containing 0.5 M NaCl (Equilibration buffer). After washing the unbound protein bound α-mannosidase was eluted with 0.4 M α-methyl-D-mannopyranoside in the same equilibration buffer at 10 mL/h. The elution of protein is monitored by checking absorbance at 280 nm as well as by checking the enzyme activity.

4A.2.7. Gel exclusion chromatography on sephacryl S-200 column

The α-mannosidase activity rich fractions from the Con A column were concentrated using centricon and applied onto a Sephacryl S-200 HR column (1 x 70 cm) previously equilibrated with 25 mM Tris-HCl pH 7.4, containing 150 mM NaCl. The protein was eluted at a flow rate of 10 mL/h. Fractions of 1ml was collected. The fractions that contain α-mannosidase activity were pooled and concentrated using Millipore Centriplus YM-30. This enzyme is used for all studies carried in this chapter.

4A.2.8. Protein estimation

Protein concentrations were determined by using commercially available Bradford dye reagent (from sigma), using BSA as the standard [Bradford, M. M., 1976].

4A.2.9. Estimation of carbohydrate content

The neutral sugar content of the purified enzyme preparation was determined by phenol sulfuric acid method of Dubois *et al.*, [1956] using glucose as standard.

4A.2.10. Native molecular weight determination

Native molecular weight of purified *M.oleifera* was determined using Sephacryl S-200 HR column (1 x 70 cm) size exclusion chromatography, according to the method described by Andrews [1964] Before loading the protein sample the column was calibrated with proteins of known molecular weight viz., Catalase (250 kDa), Alcohol Dehydrogenase (150 kDa), Phosphorylase (96 kDa), BSA (66 kDa). The protein was eluted at a flow rate of 10 mL/h. Fractions of 1ml was collected. The protein elution profile was monitored by absorbance by A_{280}. The graph was plotted as log MW versus V_e/V_o. Where: V_0 - Void volume, Ve - Protein elution volume.

4A.2.11. SDS-PAGE and sub unit molecular masses

To check the homogeneity and determine subunit molecular weight, the purified enzyme was analyzed using SDS-PAGE (stacking gel 5% and separating gel 10%) under reducing and nonreducing conditions [Laemmli U. K. 1970], using Fermentas unstained markers as standards. The gels were stained with Coomassie Brilliant Blue R-250. The subunit molecular mass of the purified α-mannosidase was determined according to Weber and Osborn [1969]. Relative mobility (R_f) of the denatured proteins on the gel was calculated as per formula given below. The molecular mass of the α-mannosidase was determined by interpolation from a linear semi logarithmic plot of log molecular mass of standard markers versus R_f values (Relative mobility).

$$\text{Mobility} = \frac{\text{Distance of protein migration x Length before staining}}{\text{Length after staining x Distance of dye migration}}$$

4A.2.12. Periodic acid Schiffs staining (PAS)

To determine the carbohydrate nature of the protein qualitatively, periodic acid - schiffs staining was carried out following the method of Zacharius *et al.*, [1969], with little modifications. Ready to use Schiffs reagent was procured from SRL. SDS-PAGE gel was stained in 1% periodic acid in 3% acetic acid for one hour. The gel

was washed for one hour with water and stained in Schiffs reagent for 30 minutes in dark. It was then destained with 10% acetic acid and finally stored in 3% acetic acid.

4A.2.13. Activity staining

Activity staining was carried out according to Blom *et al.*, [2008] to determine position of the band responsible for the α-mannosidase activity in native gels. The native PAGE was run with identical samples in different lanes. The bands corresponding to the silver staining in the native PAGE was cut into pieces and incubated in an eppendorf with substrate and performed enzyme activity under standard conditions. By comparing the part of the gel that showed the enzyme activity with the stained gel, the position of the enzyme was identified in the gel.

4A.2.14. Effect of pH and pH stability

The effect of pH on enzyme activity was determined at 37°C within a pH range of 2 to 8, using 0.1 M Citrate buffer (pH 2-3), 0.1 M NaOAc (pH 4-5), 0.1 M Sodium phosphate (pH 6-7), 0.1 M Tris-Hcl (pH 8). Stability of enzyme at various pH is determined by incubation the enzyme at various pH ranging from pH 2-8 at 37°C for 12 h. After incubation the residual enzyme activity was subsequently assayed under standard assay conditions.

4A.2.15. Effect of Temperature and Thermal Stability

Determination of optimum temperature for the *M.oleifera* α-mannosidase was performed with *p*-nitrophenyl-α-D-mannopyranoside (5 mM) in 100 mM NaOAc buffer pH 5.0 using incubation temperatures in the range of 30°C to 90°C. Thermal stability was determined by incubating the enzyme at 50°C, 60°C, 70°C, 80°C for 60 min, an aliquot was drawn at regular interval and immediately cooled. The residual enzyme activity was determined by standard assay method.

4A.2.16. Effects of EDTA and metal ions

The effect of EDTA and various divalent metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, CO^{2+}, Cu^{2+}, Hg^{2+}, Ag^{2+}, Fe^{2+}) on the activity of the α-mannosidase was carried according to Bloom *et al.*, [2008]. The effect of different metal ions on α-mannosidase activity was tested both with and without removal of preexisting ions by EDTA treatment. After EDTA treatment the enzyme is dialysed against NaOAc buffer pH 5.0. The effect of metal is tested by pre-incubating the enzyme in 100 mM NaOAc buffer pH 5.0

containing 1 mM concentration of each metal ion for 1 h prior to addition of the substrate (for EDTA 10 mM), and the residual enzyme activity was assayed by standard method. The enzyme activity without any metal ion is considered as a control (100%).

4A.2.17. Effect of other chemical agents

The effect of product analogues (mannose), simple sugars, reducing agents, β-mercaptoethanol, DTT, anionic detergent SDS, on enzyme activity was determined by pre-incubating the enzyme in sodium acetate buffer pH 5.0 containing at 37°C for 30 min. Later the remaining enzyme activity was assayed. The enzyme activity without any chemical agent is considered as a control (100%).

4A.2.18. Substrate specificity

The relative substrate specificity of α-mannosidase towards various synthetic substrates was determined. Substrates were prepared in 100 mM sodium acetate buffer pH 5.0 at final concentration of 5 mM (stock solution). The reaction was carried out using fixed concentration of enzyme and substrate under standard assay conditions. Relative activity on various substrates is expressed as percentage of the activity calculated with *p*-nitrophenyl α-D-mannopyranoside as a substrate (100%) with which enzyme showed maximum activity.

4A.2.19. Kinetic parameters determination

The Michaelis–Menton kinetic parameters (K_M and V_{max}) were determined by incubating the enzyme at optimum temperature/pH with different concentrations of substrate.

4A.2.20. Chemical modification of tryptophan residue using *N*-bromosuccinimide (NBS)

Chemical modification studies were carried out to get information about the tryptophan involvement in the active site of the enzyme. The tryptophan modifier, NBS 10 mM, (prepared in 100 mM sodium acetate buffer pH 5.0 was added in increments of 2 µL each time to the purified enzyme (0.3 mg/mL). After addition of this the residual enzyme activity was determined using the standard enzyme assay. The changes associated with the steady-state fluorescence of α-mannosidase due to NBS modification was monitored both in presence and absence of substrate (α-

methyl-D-mannopyranoside) using Perkin Elmer LS 55 fluorescence spectrophotometer, excitation at 280 nm and emission spectra was recorded in the range of 310-450 nm. The fluorescence spectra were measured at room temperature with a 1-cm path length cell. The monochromator slit width was kept at 1.5 nm in excitation and emission measurements.

4A.2.21. Effect of mannosidase specific inhibitors on the activity of α-mannosidase from *Moringa oleifera* seeds

To determine the class to which *M.oleifera* α-mannosidase is belongs two mannosidase specific inhibitors were used. Effect of two inhibitor were tested Deoxymannojirimycin (DMNJ) and Swainsonine (SW) a class I and class II inhibitors respectively, by incubating the enzyme with these inhibitors for 30 min and the residual enzyme activity was determined using standard assay.

4A.2.22. Inhibitory studies using transition state analogs

Preparation of glycoconjugates (inhibitors) and enzyme assay was carried according to Kumar *et.al*, [2009] using C1-/C2-aromatic-imino-glyco-conjugates of D-galactose, D-mannose and D-glucose (Glu2SI-Glucosyl Salicilyl Imine, GSI-Galactosyl Salicilyl Imine, GNI-Galactosyl Naphthyl Imine, Glu2NI-Glucosyl Naphthyl Imine, MNI-Mannosyl Naphthyl Imine) taking 50 µL of accordingly diluted purified enzyme with increasing amounts of the glycosidase inhibitor (i.e.; from 0 to 3 mM final conc.) at 37°C for 20 min. Enzyme assay was performed under standard assay condition. The activity without the inhibitor was considered as a 100% and the remaining activities at each concentration of inhibitor were determined w.r.t this value.

4A.2.23. Immunological studies

Ouchterlony double immunodiffusion was performed with 1% agarose gel in PBS as described by Ouchterlony [1948]. The agarose was poured into glass slide. Wells were punched in the agarose. In one well *M.oleifera* α-mannosidase was loaded and in the other well jack bean α-mannosidase antibody (available in our lab). A precipitin band was allowed to develop at 37°C for 24-48 h in humidified chamber.

4A.3.0. Results

The Glycosidase activities in the crude Tris-HCl buffer pH. 7.4 extract of *M.oleifera* seeds were examined using various *p*NP-glycosides as substrates. As shown in the Table: 4A.1, among various glycosidase it mainly contains α-mannosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and β-hexosaminidase.

4A.3.1. Purification of α-mannosidase

The purification of α-mannosidases from *M.oleifera* seeds is summarized in Table: 4A.2. Nearly 36.6 fold purification with 6.2% yield was obtained through this purification process. The crude extract was concentrated using ammonium sulphate 0-40% and 40-80%. *M.oleifera* α-mannosidase activity was observed in the second step of ammonium sulphate precipitation (40-80%). After dialysis and loaded on to DE-52 column (anion exchanger) it was observed that α-mannosidases from *M.oleifera* is completely retained on DE-52 column in Tris-HCl buffer at pH 7.4. After washing the unbound proteins the α-mannosidases was specifically eluted in stepwise manner by including NaCl in the Tris-HCl buffer pH 7.4. In DE-52 column the highest α-mannosidase activity was seen in the 100 mM NaCl elution fraction [Figure: 4A.1-A]. This fraction is used for further purification of the α-mannosidases. The enzyme rich fractions when concentrated and passed through phenyl Sepharose column (hydrophobic chromatography), enzyme is retained at high concentration of ammonium sulphate and eluted in absence of this salt [Figure: 4A.1-B]. Partially purified *M.oleifera* α-mannosidase was found to be a glycoprotein confirmed from its ability to bind to Con A-Sepharose 4B gel, When α-mannosidase rich fractions of the phenyl- Sepharose chromatography was loaded on to the Con A Sepharose *M.oleifera* α-mannosidase was strongly bound, which is later eluted specifically by using 0.4 M methyl-α-mannopyranoside in 25 mM Tris-HCl pH 7.4 containing 0.5 M NaCl [Figure: 4A.2-A]. Con A eluted enzyme rich fractions are pooled, concentrated and was further purified to homogeneity using S-200 gel filtrations, which give a one major symmetrical peak of α-mannosidase activity coinciding with protein peak [Figure: 4A.2-B]. The same column is also used for determining the native molecular mass of the intact enzyme after calibrating the column with standard proteins of known molecular weights.

4A.3.2. Molecular properties of the *M.oleifera* α-mannosidase

The purified native *M.oleifera* α-mannosidase exhibited a molecular mass of ~230-240 kDa on S-200 gel filtration [inset Figure: 4A.2-B]. In 10% SDS-PAGE, the enzyme dissociated into two subunits with molecular masses of 66 kDa (α-larger subunit) and 55 kDa (β-smaller subunit), under both reducing and non-reducing conditions, calculated with relative migration compared to the protein markers [Figure: 4A.3-A & B]. An additional band at 116 kDa was also observed which has been discussed in the later section. Under the PAS staining of the SDS-PAGE run gel it was observed that only the larger subunit got stained with the Schiffs reagent indicating, larger subunit got glycosylated. The activity assay for the native PAGE confirms the band responsible for the α-mannosidase activity [Figure: 4A.4-A, B, C & D]. The purified α-mannosidase was found to be a glycosylated protein with 9.3% carbohydrates as estimated by phenol sulphuric method.

4A.3.3. Biochemical properties of *M.oleifera* α-mannosidase

The purified *M.oleifera* α-mannosidase showed optimal enzyme activity at pH 5.0 [Figure: 4A.5-A]. Purified α-mannosidase from *M.oleifera* showed stability from pH 3 to 7 with more than 80% of the enzyme activity remained at this pH, after 12 h of incubation [Figure: 4A.5-B]. The temperature optimum of this enzyme is at 50°C. [Figure: 4A.5-C]. More than 80% of activity is seen from 40 to 60°C. Thermostability of enzyme was examined by incubation the enzyme at various temperatures and the residual enzymatic activity was measured at regular intervals for 60 min. As it can be observed from the [Figure: 4A.5-D], the result indicate more than 70% of the activity is retained after incubation at 50°C for 60 min. Enzyme completely lost its activity at 80°C after 1h of incubation and only 26%, and 12% activity is remained at 60 and 70°C, respectively.

4A.3.4. Effect of various metal ions, sugars and chemical reagents

The effect of different divalent cations and EDTA on α-mannosidase activity was tested both with and without removal of pre-existing ions by EDTA treatment. The results are summarized in Table: 4A.3. EDTA treatment leads to 56 % loss of the activity after 1 h of incubation in the acidic medium. Prolonged incubation leads to complete loss of the activity. This activity is completely restored by Zn^{2+} only, in EDTA treated samples. In presence of Cu^{2+}, Hg^{2+} and Ag^{2+} 97%, 90% and 92% of the

enzyme activity was lost respectively, in non-EDTA treated samples. However, other metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} did not show any significant loss of activity. Effect of simple sugars (mannose, glucose and galactose), reducing agents (β-mercaptoethanol and DTT) and detergent (SDS) on the activity of the α-mannosidase was examined using purified enzyme [Table: 4A.4]. The influence of the anionic detergent like SDS showed 100% inhibition on the enzyme activity at 1 mM concentration. 73% of the original activity was remained at 10 mM concentration of mannose, being a product analog it showed significant inhibition at higher then 50 mM concentration. Glucose and galactose showed 86% and 92% of the enzyme activity at 10 mM concentration. In presence of reducing agent like β-mercaptoethanol (1% v/v) light increase in the activity of about 10% was observed. DTT showed considerable decrease up to 27% in the enzyme activity at 1 mM concentration.

4A.3.5. Substrate specificity

The specificity of purified α-mannosidase was assayed in presence of synthetic substrates. No significant activities towards other para-nitrophenyl- glycosides were observed except *para*-nitrophenyl-α-D-mannopyranoside [Table: 4A.5]. The K_m and V_{max} value was measured using *p*-nitrophenyl-α-D-mannopyranoside and was found to be 1.6 mM and 2.2 U/mg respectively.

4A.3.6. Chemical modification studies

The α-mannosidase activity was completely inhibited at 1 mM of NBS. Modification of tryptophan residue by NBS resulted in total quenching of fluorescence. Modification of tryptophan residue by NBS also associated with the blue shift quenching of the fluorescence spectrum [Figure: 4A.6-A]. However, in presence of substrate (α-methyl-D-mannopyranoside) total quenching was protected [Figure: 4A.6-B].

4A.3.7. Effect of mannosidase specific inhibitors

The effect of mannosidase specific inhibitors such as deoxymannojirimycin (DMNJ) and swainsonine (SW) on enzyme activity is shown in Table: 4A.6. The enzyme activity was completely inhibited by SW at 0.001 mM (1 µM) concentration. Whereas, DMNJ at the same concentration did not influence the enzyme activity. At 0.5 mM concentration of DMNJ showed only 31% inhibition.

4A.3.8. Inhibition by glycoconjugates

The inhibition results of the glycoconjugates are shown in [Figure: 4A.7-A]. Among the tested different glycoconjugates naphthylidene-conjugates of mannose, glucose and galactose exhibit 100% inhibition at 1.0 mM, 1.5 mM, and 2.0 mM concentration respectively. The salicylidene-conjugates of the same sugars exhibit 100% at 3.0 mM concentration. Concentration at which a 50% inhibition of enzyme is brought (IC_{50}) by glyco-conjugates is shown in Figure: 4A.7-B.

4A.3.9. Immunochemical study

In order to investigate immunological relationships of the *M.oleifera* α-mannosidase with that of the jack bean α-mannosidase, Ouchterlony double immunodiffusion analysis was carried using antisera raised against jack bean α-mannosidase. An arc was observed between the two wells indicating that *M.oleifera* α-mannosidase cross-reacted with jack bean α-mannosidase antisera [Figure: 4A.8].

FIGURES

Figure: 4A.1-A & B.

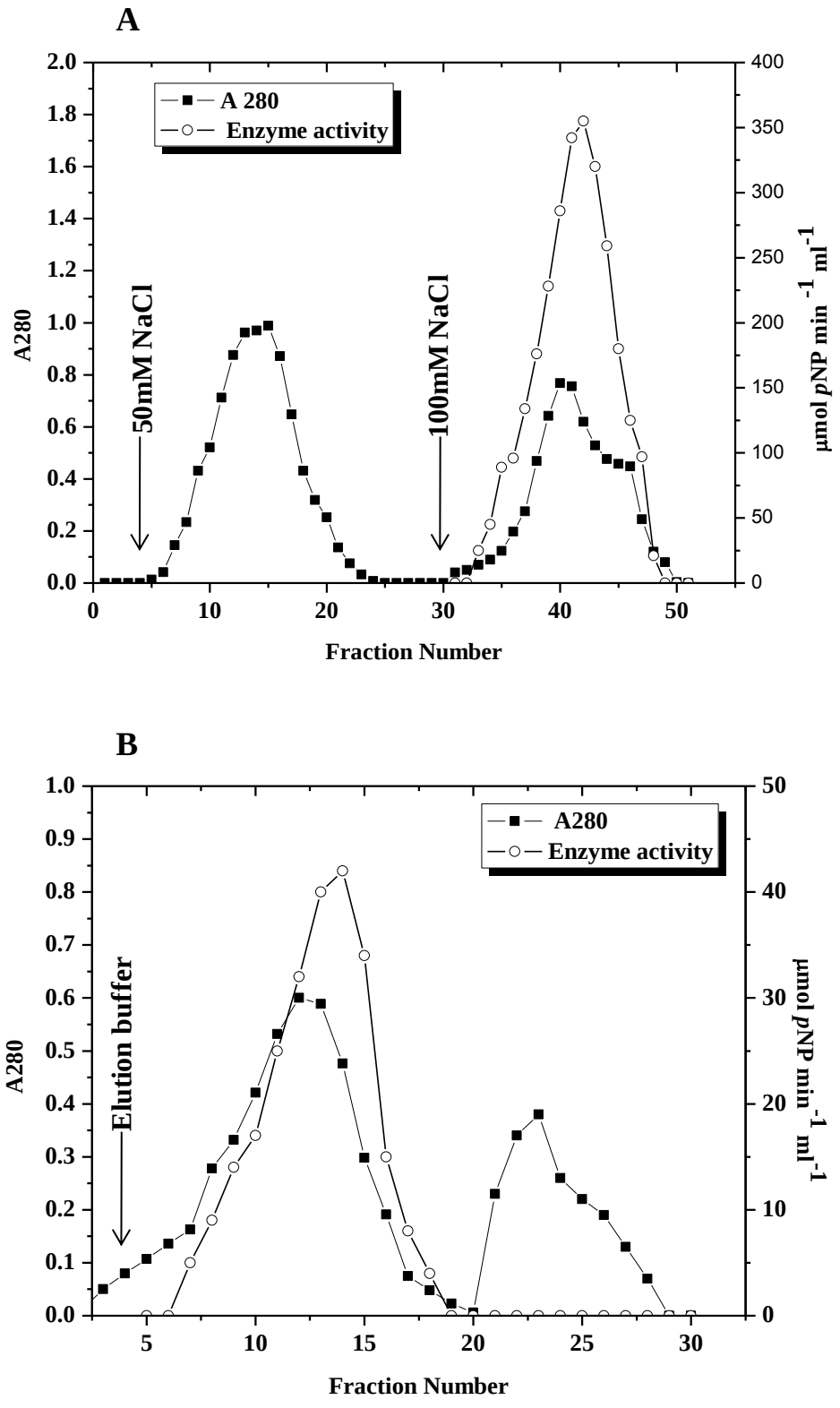


Figure: 4A.1 A & B. Elution profile of *M.oleifera* α-mannosidase on (A) DE-52 column and (B) phenyl Sepharose column: (A) The crude enzyme fraction of *M.oleifera* seeds after $(NH_4)_2SO_4$ precipitation and dialysis was applied onto the DE-52 column (4 cm X 9 cm). The column was washed with 25 mM Tris-HCl buffer pH 7.4 (equilibration buffer), and the bound enzyme was eluted by 100 mM NaCl in the same buffer. Fractions of 1 mL were collected and the absorbance monitored at 280 nm. Arrow indicates point of application of salt. **(B)** The enzyme rich fractions from the DE-52 were concentrated and saturated to 1 M $(NH_4)_2SO_4$ in equilibration buffer. After washing, the bound protein was eluted with buffer in the absence of $(NH_4)_2SO_4$.

Figure: 4A.2 A & B

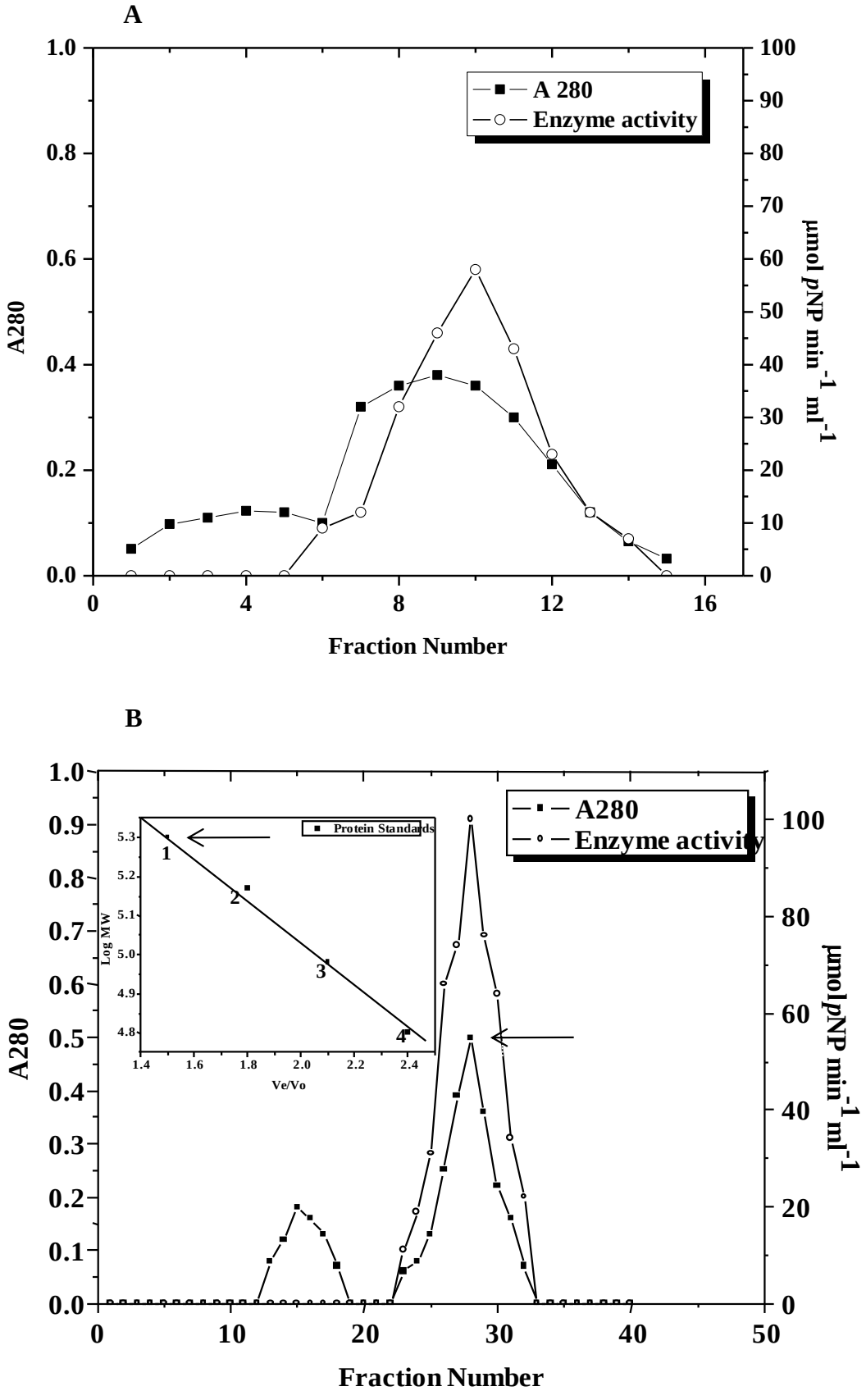
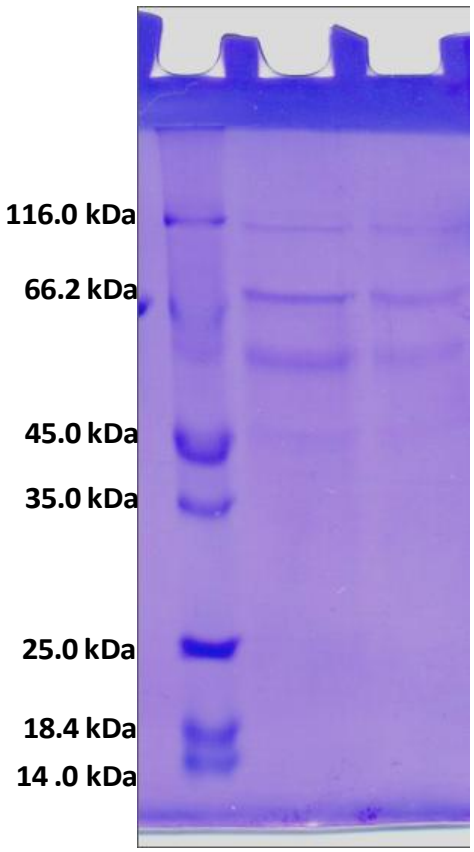
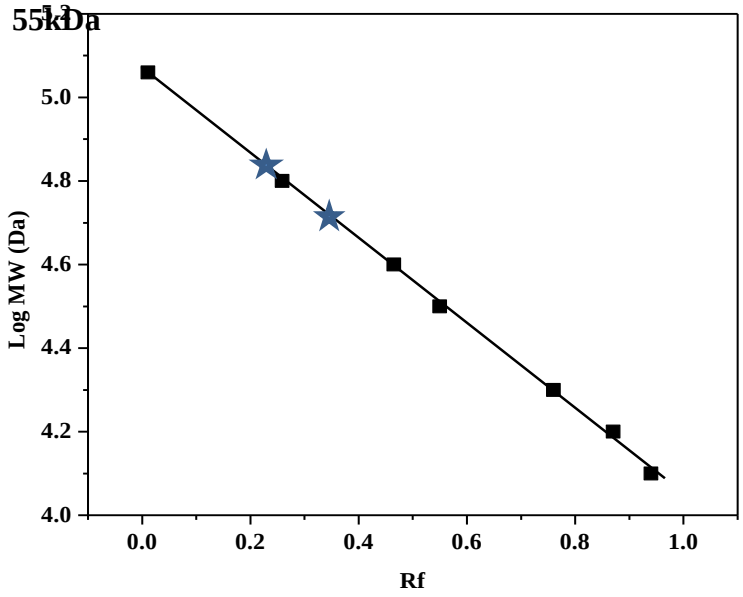


Figure: 4A.2A & B. (A) Elution profile of α-mannosidase from Con A column and (B) Gel filtration of the enzyme on S-200 gel and molecular weight determination: (A) DE-52 enzyme rich fractions were pooled and loaded on to the Con A-Sepharose 4B column (5 mL) previously equilibrated with 25 mM Tris-HCl pH 7.4 containing 0.5 M NaCl (Equilibration buffer). After washing the gel with equilibration buffer, the bound α-mannosidase was eluted with 0.4 M α-methyl-D-mannopyranoside in the same buffer. **(B)** The α-mannosidase eluted from Con-A gel was concentrated and loaded onto a Sephacryl S-200 column (1 x 70 cm). The protein was eluted at 10 mL/h with 25 mM Tris-HCl buffer pH 7.4 containing 150 mM NaCl. The eluted fractions were checked for the enzyme activity. The same column was also calibrated using standard proteins of known molecular weights 1.Catalase (250 kDa), 2. Alcohol Dehydrogenase (150 kDa), 3. Phosphorylase (96 kDa) and 4. BSA (66 kDa). Ve/Vo values were plotted against Log molecular weight of the protein. Arrow indicates the point where *M.oleifera* α-mannosidase was eluted.

Figure: 4A.3A & B.



A



B

Figure: 4A.3 A&B.

A) **10% SDS-PAGE of purified *Moringa oleifera* α-mannosidase:** Lane M: Standard molecular weight markers, Lane 1 & 2: Purified α-mannosidase under both reducing and non reducing conditions respectively. Gels were stained with Coomassie Brilliant Blue. The standard protein molecular weight markers used were; β- galactosidase (166.0 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), lactate degyhydrogenase (35.0 kDa), REase Bsp 981 (25 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa) (Fermentas).

B) **Determination of Subunit molecular weights of *Moringa oleifera* α-mannosidase:** The mobility of the protein was determined as per the formula given in the text. The values are plotted against the log MW (Da) Vs R_f values.

Figure: 4A.4A, B, C & D

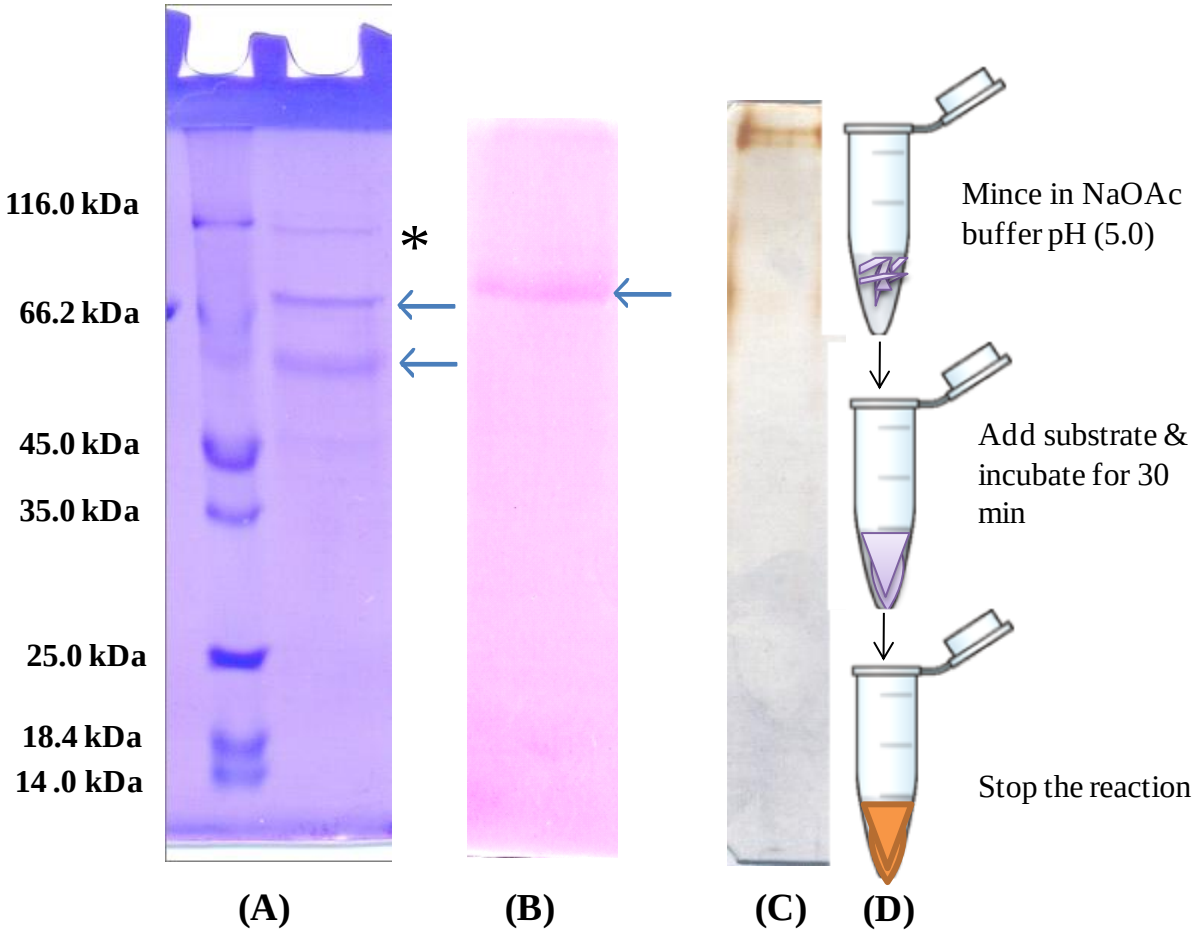


Figure: 4A.4A, B, C & D. (A) 10 % SDS-PAGE , (B) Periodic acid schiffs (PAS) staining, (C) Native PAGE activity staining and (D) Activity assay of *M.oleifera* α-mannosidase: (A) 10% SDS-PAGE of purified *M.oleifera* α-mannosidase (showed for comparison). **(B)** The same gel was run in duplicates under reducing conditions and stained with Schiffs reagent (PAS staining). Arrow corresponds to the larger subunit (66 kDa) protein in the SDS-PAGE. **(C)** Native-PAGE of the purified *M.oleifera* α-mannosidase used for the activity staining. **(D)** The corresponding band of the native - PAGE run in duplicate was used for activity assay after incubation with both buffer and substrate for 30 min reaction was stopped and the color change observed.

*Indicates possible 116 kDa precursor form of mannosidase containing mannose binding domains. (As identified with MALDI-TOF; MS/MS analysis)

Figure: 4A.5. A, B, C & D.

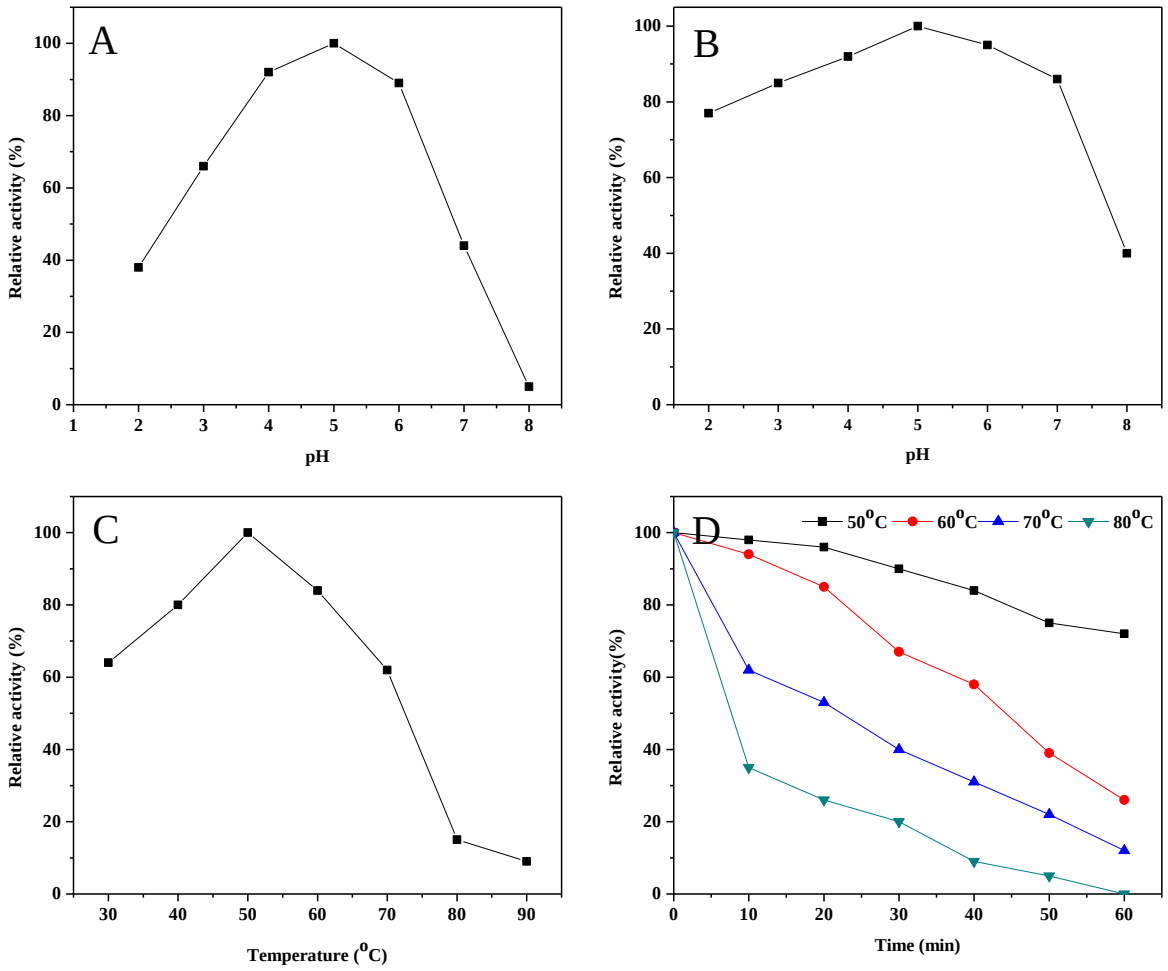


Figure: 4A. 5. A, B, C & D. Characterization of the enzymatic properties of purified α-mannosidase from *Moringa oleifera*. (A) Effect of pH on the α-mannosidase activity of *M.oleifera* was determined at 37°C in buffers ranging from pH 2.0 to 8.0. The value obtained at pH 5.0 where α-mannosidase activity is maximum was taken as 100%. **(B)** pH stability of *M.oleifera* α-mannosidase was determined by measuring α-mannosidase activity under standard assay conditions (*p*NPM) after pre-incubation of the enzyme at 37°C for 12 h in buffers ranging from pH 2.0 to 8.0. The activity of an untreated enzyme sample at pH 5.0 was taken as 100%. **(C)** Effect of temperature on α-mannosidase activity was determined in 100 mM NaOAc buffer (pH 5.0) at 30–90°C. The value obtained at 50°C was taken as 100%. **(D)** Thermostability of α-mannosidase was determined by measuring α-mannosidase activity under standard assay conditions after pre-incubation of the enzyme in 100 mM NaOAc buffer (pH 5.0) at 50, 60, 70 and 80°C for various periods. The activity of an unheated enzyme sample was taken as 100%. Data is the mean of triplicate experiments.

Figure: 4A.6 A & B

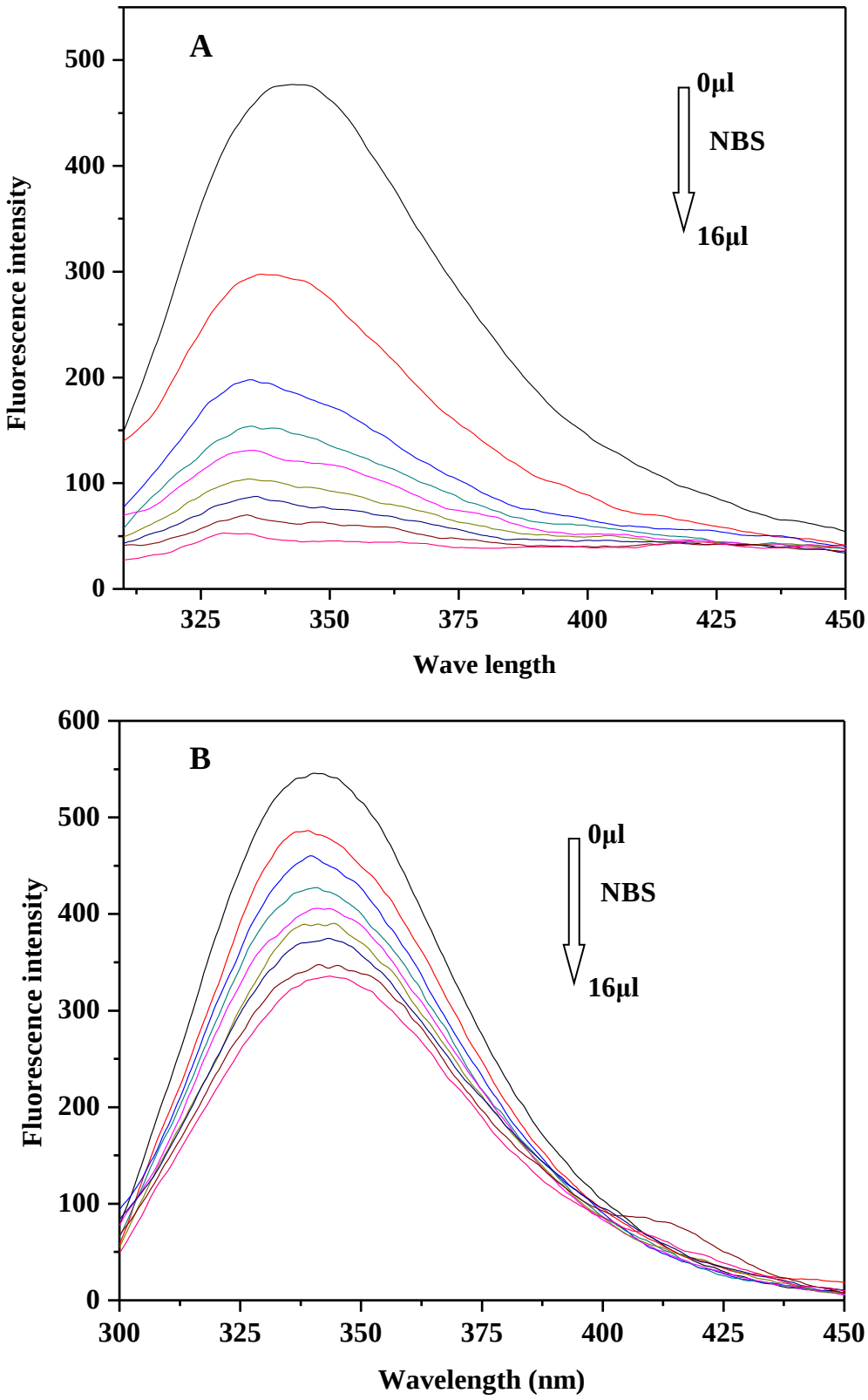
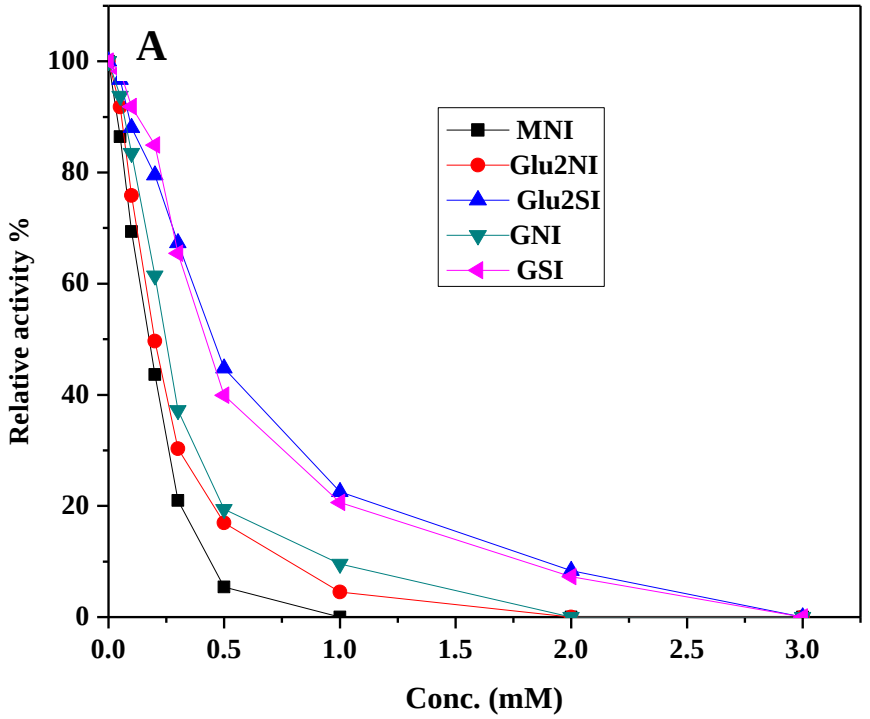


Figure: 4A.6 A & B. Influence of NBS on *Moringa oleifera* α-mannosidase activity: (A) Fluorescence spectra of *M.oleifera* in absence and **(B)** in presence of substrate. 10 mM NBS (prepared in 100 mM sodium acetate buffer pH 5.0 was added each time in the increment of 2 μL each time. The Fluorescence was recorded by excitation at 280 where, maximum emission was observed and emission was recorded in the range of 310-450 nm.

Figure: 4A.7 A & B



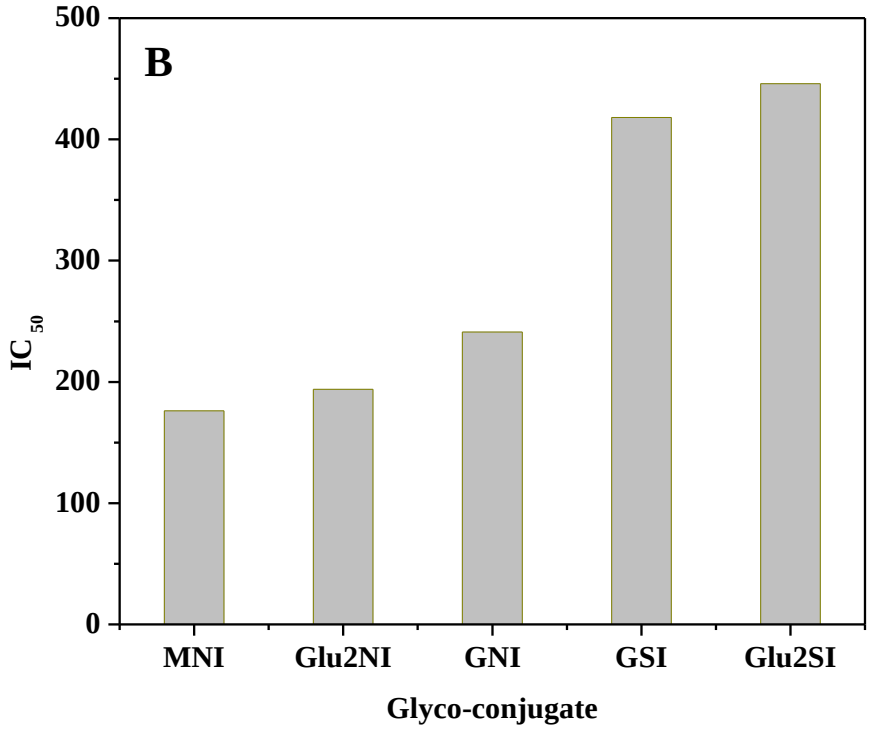


Figure: 4A.7 A & B. (A) Inhibition of enzyme activity with different Glyco-conjugates, (B) IC_{50} values: (A) C1-/C2-aromatic-imino-glyco-conjugates of D-galactose, D-mannose and D-glucose: the purified α-mannosidase from *M.oleifera* was incubated with these glyco-conjugates for 20 min. The structures of the synthetic glyco-conjugates used in this study are given in figure.3.4. Enzyme assay was performed using standard assay conditions. The activity without the inhibitor was considered as a 100%. The relative activity at each concentration was measured. **(B)** IC_{50} values of these glyco-conjugates. Each value is mean of three independent readings.

Figure: 4A.8

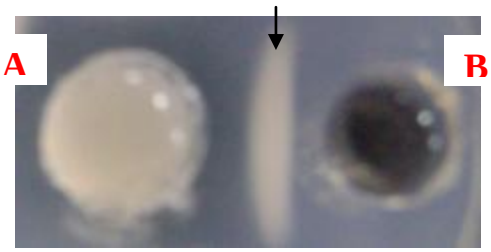


Figure: 4A.8. Immunological cross-reactivity of *Moringa oleifera* α-mannosidase with the Jack bean α-mannosidase antiserum: Well A: Jack bean α-mannosidase antibody and Well B: *Moringa oleifera* α-mannosidase. Arrow indicates the precipitin line (Arc).

TABLES

Table: 4A.1. Various glycosidase activities in the crude *M.oleifera* seeds extracted in 25 mM Tris-

Glycosidases	Enzyme activity (U/mg)[a]
α-mannosidase	28.7
α-galactosidase	29
β-galactosidase	14
α-glucosidase	20
β-glucosidase	16
β-hexosaminidase	13.7

[a] The enzyme units were calculated for 1g of the seed tissue. The enzyme activities were measured at pH 5.0 using *p*NP-glycosides (5 mM) as substrates as described under methods.

Table: 4A.2. Purification of α-mannosidase from *Moringa oleifera* Seeds

Purification step	Total Protein (mg)	Total activity [b] (units)	Specific activity [c]	Yield (%)	Fold purification
Crude extract [a]	1440	1435	0.99	100	1
40-80% Ammonium sulfate	520	1224	2.3	85.2	2.3
DE-52 cellulose	120	846	7	58.5	7.07
phenyl-Sepharose CL-6B	15	425	28.3	29.6	28.5
Con-A Sepharose	7	220	31.4	15.3	31.7
Sephacryl- S 200	2.5	90	36	6.2	36.6

[a] 50 g of defatted *Moringa oleifera* seed kernel powder was used at a time and the purification in different chromatographic steps was carried out in batch wise. Proteins were measured in chromatographic fractions using A_{280}.

b One unit of enzyme activity was defined as amount of enzyme hydrolyzing 1 µmol of *p*-nitrophenol from PNPG per min at pH 5.0 and 37°C.

c The specific activity of the enzyme is expressed as units per mg protein. (U/mg of protein).

Table: 4A.3. Effect of divalent metal ions and EDTA on the *Moringa oleifera* α-mannosidase activity.

Effector Agent	**Relative Activity (%)**	
	Non-EDTA treated	EDTA treated
Control	100	100
EDTA [a]	44	-----
Ca^{2+}	97	96
Mg^{2+}	92	81
Mn^{2+}	86	83
Zn^{2+}	101	203
Fe^{2+}	65	78
Cu^{2+}	3	0
Co^{2+}	36	45
Hg^{2+}	10	0
Ag^{2+}	8	0

[a] EDTA concentration 10 mM, all other metal ions 1 mM, untreated enzyme with metal ion is taken as control 100%.

Table: 4A.4. Effect of simple sugars, denaturing and reducing agents on *M.oleifera* α-mannosidase activity.

Chemical agent	Concentrations	Relative activity (%)[a]
Simple sugars		
Mannose	5mM	85
	10mM	73
	50mM	30
Glucose	5mM	94
	10mM	86
	50mM	46
Galactose	5mM	97
	10mM	92
	50mM	83
SDS (Anionic detergent)	1mM	ND
Reducing agents		
β-mercaptoethanol	1% (v/v)	110
DTT	1mM	73

[a] Relative activities are measured taking enzyme activity of *M.oleifera* α-mannosidase without adding any chemical agent as 100%. All the experiments are performed in triplicates and the mean values are represented. ND: Not Detected

Table: 4A.5. Relative Activity of *M.oleifera* α-mannosidase with various synthetic substrates.

Substrate [a]	Relative activity (%) [b]
***p*NP-α-D-mannopyranoside**	100
***p*NP-α-D-glucopyranoside**	0.22
***p*NP-β-D-glucopyranoside**	0.18
***p*NP-β-D-galactopyranoside**	0.12
***p*NP-α-D-galactopyranoside**	0.09

[a] All the synthetic substrate concentration used were 1 mM in the final reaction mixture.
[b] Relative activities were calculate in relation to *pNP*-α-D-mannopyranoside activity that was considered as 100%.

Table: 4A.6. Effect of mannosidase specific inhibitors on the activity of α-mannosidases from *Moringa oleifera* seeds.

Inhibitors	Concentration(mM)	Relative activity (%)
Control	0	100%
Swainsonine (Class II)	0.001	5%
	0.01	ND
	0.05	
	0.1	
	0.5	
Deoxymannojirimycin (Class I)	0.001	100
	0.01	99
	0.05	97
	0.1	78
	0.5	69

Results were the percentage relative activity compared to the control which is taken as 100%. ND: Not Detected.

4A.4.0. Discussion

In the frame work of our interest for new glycosyl hydrolases from the seeds of non-legumes we have successfully purified a class II α-mannosidase from *M.oleifera* seed kernels using conventional chromatographic techniques. To our knowledge there have been no reports on the purification of any glycosidase from *M.oleifera* seed kernels. The purification protocol involved four chromatographic steps anion exchange chromatography, hydrophobic interaction chromatography, Con-A lectin affinity chromatography, and S-200 gel permeation. In the first step of ammonium sulphate precipitation (0-40%) no detectable α-mannosidase activity was observed. This fraction contained largely small cationic proteins, which are abundant in *M.oleifera* [Gassenschmidt *et al.,* 1995]. The α-mannosidase activity was mainly observed in the 40-80% fraction. On DE-52 this enzyme is completely retained and was eluted with increasing concentration of the NaCl in the buffer. The step wise elution of the enzyme from the DE-52 also helps in separating the α-mannosidase from other contaminating proteins such as the α-galactosidase which, appears in the crude extract. The fact that the enzyme binds strongly to Con-A gel suggests its glycoprotein nature which is further confirmed by its specific elution from the gel using the sugar methyl-α-mannopyranoside. The single protein peak which overlaps with the enzyme activity peak suggests that it is a homogeneous preparation. The native molecular mass of the purified enzyme was approximately 230 kDa as determined by gel filtration. Native PAGE analysis of the enzyme revealed that it is homogeneous and the band exhibited enzyme activity. On the other hand, by SDS-PAGE under reducing and non reducing conditions the enzyme dissociated into two subunits of molecular mass 66 kDa and 55 kDa, suggesting that this enzyme to be tetrameric. It is comparable to Jack bean and tomato α-mannosidase. However, the subunit masses differ from these species. Jack bean enzyme has two subunits of 66 kDa and 44 kDa, whereas, tomato enzyme has subunits of 70 kDa and 47 kDa respectively [Snaith, 1975, Hossain, *et al.,* 2009]. Under both reducing and non reducing conditions the enzyme showed similar band pattern suggesting the interaction between the subunits to be non-covalent which is common feature as reported earlier [Tulasi and Nadimpalli, 1997; Kimura *et al.,* 1999; Mahadevi, *et al,* 2002; Hossain, *et al.,* 2009]. Monomeric forms of mannosidase were also observed in some plant species like *Artocarpus communis* seeds (Isoform I-75 kDa; II-61 kDa),

Erythrina indica seeds (127 kDa) [Kestwal and Bhide, 2005; Ahi *et al.,* 2007]. However *Ginko biloba* seeds α-mannosidase is 340 kDa in the native form and subunit of 120 kDa [Woo *et al.,* 2004]. In closely related members of *M.oleifera* order like tropical fruit babaco (brassicales) oligomeric form of α-mannosidase was observed [Blom *et al.,* 2008]. *Prunus serotina* Ehrh and *Medicago sativa* [Curdel and Petek, 1980; Waln and Poulton, 1987] also has the four-subunits with different molecular masses. *M.oleifera* is a glycoprotein as detected by periodic acid-Schiff staining. This was further confirmed to contain 9.3 % carbohydrate. This carbohydrate content is more than that of mannosidase from *Erythrina indica* seeds [Kestwal, *et al.,* 2007]. *M.oleifera* α-mannosidase when stained with PAS staining, it was observed that only the larger subunit got stained with the Schiffs reagent [Figure: 4A.4-B] indicating, larger subunit is glycosylated, it is also observed with jack bean α-mannosidase [Kimura *et al.,* 1999]. The purity of the α-mannosidase was verified by native PAGE (silver staining) and showed one homogeneous band [Figure: 4A.4-C]. The enzymatic identity of this band was verified by cutting identical unstained lanes of the same gel, equivalent to band in Figure: 4A.4-C, into different pieces and performing activity assays. By relating the presence of activity with the respective position on the gel, the band responsible for the α-mannosidase activity could be identified Figure: 4A.4-D. SDS-PAGE showed one additional band at 116 kDa region, which was consistently seen in all the purification steps. This protein band when subjected to MALDI analysis for the identification of the protein, it was confirmed to be 116 kDa glycoprotein containing a mannose binding domains in it [results not shown]. This results guide to interesting findings which are reported on class II α-mannosidase [Howard *et al.,* 1998]. In humans lysosomal α-mannosidase the initial enzyme is synthesized as a polypeptide of 110 kDa that is subsequently processed into two subunits of 40–46 kDa and 63–67 kDa, which then constitute the native protein (molecular mass, 210 kDa). The rat Golgi α-mannosidase II is a dimer composed of 124 kDa subunits. Treatment with chymotrypsin causes limited proteolysis to give a dimer of 110 kDa subunits that retains full activity. Some studies reported the possibility that the jack bean α-mannosidase is also synthesized as a polypeptide chain of 110 kDa, which forms a dimer. The fragments of mass 44 kDa and 66 kDa, observed by SDS-PAGE, could be due to limited proteolysis of the protein, which is still able to maintain its integrity unless exposed to denaturing conditions [Howard *et al.,* 1998].

The optimum pH of *M.oleifera* α-mannosidase enzymes was around pH 5.0 when tested with *p*NP-α-D-mannopyranoside as a substrate, similar to those of other plant α-mannosidases reported [Ahi *et al.,* 2007; Hossain, *et al.,* 2009]. Some enzymes have pH optimum between the range of pH 4.0-5.0 [Prijcker, *et al.,* 1974; Paus, 1977; Niyogi and Singh, 1988; Kishimoto *et al.,* 2001]. The optimal pH for the almond α-mannosidase was around pH 3.8 [Misaki *et al.,* 2003]. The enzyme was active at acidic pH and activity decreased as the pH approached the alkaline range pH 8.0. This is within the range of reported values of α-mannosidase from *Capsicum* and tropical fruit *Babaco* [Priya Sethu and Prabha, 1997; Blom *et al.,* 2008]. It is interesting to note that the activity of the glycosidases is high at acidic pH and the pH that naturally exists in the protein bodies of the seeds where these enzymes are co-localized with storage proteins, lectins. This suggests possible *in vivo* physiological significance of their co-localizations that may be important during seed growth and development.

The optimum temperature of this α-mannosidase was found to be 50°C, above this temperature, enzyme activity declined rapidly as the temperature increased, but the enzymes were not completely inactivated even at 80°C after 30 min of incubation. Further the thermal stability was studied by incubating the enzyme at different temperatures for 60 min. The enzyme is quiet stable to 50°C for 60 min with more than 75% of the activity retained. Even when it was incubated at 70°C for 40 min 30% of the original activity remained. This high temperature stability may be due to two reasons one is due to the tetrameric form which are stable compared to the monomeric and dimeric forms; the second reason is due to the high glycosylation of this enzyme.

Among several metal ions incubated with the EDTA treated sample, only Zn (II) is reported to restore the total activity of the enzyme. The study of inactivation of α-mannosidase by EDTA and its reversion leads to the conclusion that α-mannosidase is a zinc-containing metalloprotein, as observed for other α-mannosidases from various plant species [Curdel and Petek, 1980; Conti, *et al.,* 1987; Kishimoto *et al.,* 2001; Kestwal and Bhide, 2005; Blom *et al.,* 2008]. The role played by Zn^{2+} in the enzymatic reaction has not yet been elucidated. The zinc atoms may be involved in subunit interactions. Cu (II), Hg (II) and Ag (II) are potent inhibitors of some plant α-mannosidases [Woo *et al.,* 2004], whereas Mn (II), Mg (II) and Ca (II) have no effect on the α-mannosidases.

The effect of divalent cations is a useful parameter to distinguish among different α-mannosidases. Members of family 47 can be stimulated by Ca^{2+} ions whereas activity of class II α-mannosidases of family 38 exhibit diverse forms of metal ion dependency, cadmium activates the α-mannosidase activity in *T.maritima* [Nakajima, *et al.,* 2003] Co (II) is the preferred cofactor for the α-mannosidase from insect and *Bacillus sp.* [Kawar *et al.,* 2001; Nankai *et al.,* 2002], while the activity of the enzymes from jack bean require Zn (II) ions [Howard *et al.,* 1997].

The anionic detergent like SDS showed complete inhibition on the enzyme activity at 1 mM concentration that can be attributed for the partial or complete disruption of the higher order structures of the enzyme. About 73% of the original activity remained at 10 mM concentration of mannose, being a product analog it showed inhibition at higher concentration.

The purified α-mannosidase was checked for other glycosidase activities which are present in the initial crude extract with *p*NP-substrates such as *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-D-galactopyranoside, This α-mannosidase from *M.oleifera* seeds did not show significant activity with these substrates, this indicates that the activity of this enzyme is restricted towards *p*NP-α-D-mannopyranoside only.

Preliminary experiments were done to know the involvement of tryptophan in the active site of the enzyme as tryptophan residues are essential for substrate binding in many glycosidases, including lysozyme, glucoamylase, cellulase and xylanase. Modification of the enzyme by NBS resulted in complete loss of activity suggesting the role of tryptophan in the catalytic activity of the enzyme. When enzyme was incubated along with the substrate quenching of fluorescence is incomplete suggesting the role of tryptophan in the active site of *M.oleifera* α-mannosidase. Some studies showed that addition of a substrate like *p*NP-α-D-mannopyranoside to the enzyme prior to NBS treatment protected the enzyme [Kestwal *et al.,* 2007].

In jack bean 4 tryptophan residues are present per enzyme monomer that is involved in enzyme activity [Burrows and Rastall, 1998]. In *Canavalia ensiformis, Phaseolus vulgaris* and *Erythrina indica* seeds carboxyl and tryptophan residues present at the catalytic site are essential in enzyme activity [Paus, E. 1978; Burrows and Rastall, 1998; Kestwal *et al.,* 2007].

Plant α-mannosidases have been classified into two independently derived groups, Class I and Class II, based on inhibitor profiles [Daniel *et al.,* 1994; Eades *et al.,* 1998]. Regarding inhibitor profiles, α-mannosidases susceptible to kifunensine (KIF) and deoxymannojirimycin (DMNJ) inhibition belong to class I α-mannosidases. In contrast, those sensitive to swainsonine (SW) and 1, 4-dideoxy 1, 4-imino-D-mannitol (DIM) belong to class II mannosidases [Ahi *et al.,* 2007]. In glycosyl hydrolase classification class I α-mannosidase belongs to GH family 47 specific for α -1,2 linkage, they are activated by Ca (II) ions, whereas, class II α-mannosidase belongs to GH family 38 specific for α1,2; α1,3 ; α1,6 they are activated by Zn (II) ions.

We checked the effect of mannosidase specific inhibitors such as SW, DMNJ on the α-mannosidase activity. The activity was totally lost in presence of SW a furanose analogue at very low concentrations. While, at the same concentration DMNJ a pyranose analogue didn't show any effect.

Studies showed that at pH 5.0 the optimum pH of breadfruit α-mannosidase, SW binds strongly to the catalytic centre of the enzyme due to ionization of the SW which, in turn acts as the strong inhibitor of the enzyme [Ahi *et al.,* 2007]. In this respect the *M.oleifera* enzyme shows similar to the breadfruit α-mannosidase.

This study showed that the α-mannosidase purified from seeds of *M.oleifera* is class II α-mannosidase since it is sensitive to furanose transition state analog SW. Similar results was also observed in Jack bean, rice and bread fruit α-mannosidase [Howard *et al.,* 1997; Kishimoto *et al.,* 2001; Ahi *et al.,* 2007]. Obtaining the sequence information of this protein will give more insights into the phylogenetic relationship between different classes of α-mannosidase in a wide variety of organisms. The immunochemical results suggest *M.oleifera* α-mannosidase is immunologically similar to jack bean α-mannosidase and posses some identical antigenic determinants with that of the jack bean α-mannosidase.

The inhibition of enzyme activity caused by various synthetic glycoconjugates revealed that naphthylidene-conjugates were more potent inhibitors then the salicylidene-conjugates of the simple sugars. These results indicate that the naphthylidene-conjugates have more affinity to bind the enzyme and cause the inhibition. Similar results were reported with the Jack bean α-mannosidase [Kumar *et al.,* 2009]. It is interesting to note that the two mannosidases purified from different

plant families, legume (*Canavalia ensiformis*) and non legume (*M.oleifera*) exhibit similar sugar binding properties suggesting that they have possibly similar aminoacid sequence that permit efficient binding of bulky groups in synthetic sugars. This results states that they are highly conserved among different families of the plant kingdom.

A family of class II α-mannosidase was purified and characterized from *M.oleifera* in this present study for the first time. The enzyme was found to have heterogenous subunits of larger 66 kDa and smaller 55 kDa. Interestingly larger subunit was glycosylated with the carbohydrate content around 9.3 %. The enzyme associated with covalently bound Zn^{2+} ions. The distinctive characteristic of the *M.oleifera* α-mannosidase was its thermal stability which was not reported in other known α-mannosidase. A chemical modification study highlights the involvement of tryptophan residue at the active site of the enzyme. Mannosidase specific Inhibitor studies helped to classify this enzyme in to class II mannosidase, belonging to GH family 38. In recent years, the functional significance of the carbohydrate moieties has been increasingly appreciated. Carbohydrates covalently attached to polypeptide chains can confer many functions to the glycoprotein, for example, resistance to proteolytic degradation, the transduction of information between cells, and ligand–receptor interactions are a few to mention. Furthermore, these characteristics show the *M.oleifera* α-mannosidase importance in production of short sugar chains active biologically, and could find application in food and biotechnology industry.

CHAPTER 4B

Purification, Biochemical characterization and localization studies of acidic α-galactosidase from *Moringa oleifera* seeds

4B.1.0. Introduction

α-galactosidase (α-D-galactoside galactohydrolase EC 3.2.1.22) is widely distributed in microorganisms, plants and animals [Dey and Pridham, 1972]. It is an exoglycosidase that catalyzes hydrolysis of terminal α-1-6-linked galactosyl residues from a wide range of substrates including oligo-saccharides of raffinose family sugars; raffinose, stachyose, melibiose, verbascose and polysaccharides of galactomannans, locust bean gum and guar gum. More over it also hydrolyzes glycoconjugates; glycoproteins and glycosphingolipids [McCleary and Matheson, 1974]. In plant kingdom, α-galactosidases were reported to occur in actively growing as well as in fully developed leaves and fruits, and also in seeds and tubers [Dey and del Campilo, 1984; Keller and Pharr, 1996]. The enzyme from coffee beans was one of the first α-galactosidase to be partially purified and biochemically characterized. The α-galactosidases are classified as either acid or alkaline, according to the optimal pH at which they are active. Most of the α-galactosidases isolated from plant seeds and leaves are acidic enzymes and belong to the 27 glycosyl hydrolase cluster family [Fialho *et al.,* 2008]. Some of the isoform types of the enzyme may be distributed in the cytosol as well as the vacuole, and in some reported cases also in the cell wall [Keller and Pharr, 1996]. In plants this enzyme catalyzes the hydrolysis of the free galactose from naturally occurring galactosyl-sucrose oligosaccharides such as raffinose and stachyose, as well as other α-galactosides such as galactolipids and galactoproteins. One noted function of the enzyme is in the mobilization of α-D-galactosyl residues stored within the raffinose family of oligosaccharides (RFO) and other storage polysaccharides during germination and sprouting of seeds and tubers [Soh *et al.,* 2006]. In plant species the hydrolase usually acts together with (1→4)-*β*-mannan endohydrolases (endo-*β*-mannanase) (EC 3.2.1.78) and *β*-mannosidases (EC 3.2.1.25) to degrade GMs, mainly during germination of plant seeds [Reid and Meier, 1973]. The enzyme activity has been shown to increase during seed germination concomitant with catabolism of galactomannans and other reserve polysaccharides without which this process will be restrained [Marraccini *et al.,* 2005]. Since seeds contain large amounts of such storage compounds, which are utilized as an energy source during germination, work on plant α-galactosidases has mostly focused on seed tissues [Dey and Wallenfels, 1974; Chandra Sekhar and De Mason, 1990]. More recently, site-directed mutation experiments permitted to identify amino acid residues essential for the activity of coffee bean α-Gal [Zhu *et al.,* 1995; Zhu *et al.,* 1996;

Maranville and Zhu, 2000]. It has been known that the deficiency of *α*-galactosidase leads to the Anderson– Fabry's disease in humans [Eng *et al.*, 2001]. It is an X-linked lysosomal storage problem can be treated by α-galactosidase replacement therapy [Gieselmann, 1995]. Some of the α-galactosidases isolated from various plant and microbial sources have transglycosylation activities especially at a high concentration of substrate a catalytic property that might be relevant to cell wall modification during fruit growth and development [Chin-Pin *et al.*, 2006; Gote *et al.*, 2006]. The enzyme has attracted attention in the medical application due to its capacity to remove the terminal galactose units (α 1-3 linked) from the blood group B cell surface carbohydrate moiety of glycoprotein complexes, thus generating type O red blood cells [Kruskall *et al.*, 2000; Hata and Smith, 2004]. Furthermore, α-galactosidases had many potential biotechnological applications, including: pre-treatment of animal feed to hydrolyze non-metabolizable sugars, thereby increasing the nutritive value [Ghazi *et al.*, 2003], degradation of raffinose to improve the crystallization of sucrose [Ganter *et al.*, 1988], processing of soy molasses and soybean milk [Thananunkul *et al.*, 1976], improvement of the viscosity and gelling properties of galactomannan [Dey *et al.*, 1993], stimulation of oil/gas wells through hydrolysis of the propant matrix [McCutchen, 1996], Because of their medical and technological importance, a number of α-galactosidases from eukaryotic and microbial sources have been studied.

Recently the activities of different glycosidases such as the α-galactosidase, α-mannosidase, α-glucosidase, β-glucosidase, β-galactosidase and N-acetyl β-D-hexosaminidase were checked in the extracts obtained from the seeds of *M.oleifera* that were devoid of the lectin. When analyzed using *para*-nitrophenyl derivatives of the corresponding sugars as substrates, interestingly, α-galactosidase activities were the highest followed by the α-mannosidase activity. Other glycosidase activities were found to be significantly lower compared to the α-galactosidase. Such high activity of the α-galactosidase prompted us to purify the enzyme and to study some of its properties. This is the first time that we report on the glycosidase from the seeds of *M.oleifera*. In the present study we used different chromatographic methods to purify α-galactosidase from seed kernel and looked for its biochemical characteristics. In order to identify important residues required for activity of the enzyme chemical modification studies were carried out with this enzyme. Additionally, Subcellular localization of the *M.oleifera* α-galactosidase in the seeds was studied by isolating the protein bodies from the kernel.

4B.2.0. Materials and methods

4B.2.1. Materials

Dry *Moringa oleifera* seeds (PKM_1 variety) were obtained from local market. *p*-nitrophenyl-α-D-galactopyranoside (*p*NP-α-Gal), other *p*-nitrophenyl glycosides, phenyl Sepharose CL-4B, Sephadex G-150 was obtained from Sigma-Aldrich, CM-cellulose from Whatman. Ready to use protein molecular weight marker mixture for SDS-PAGE was obtained from Fermentas. All other chemicals and reagents were of analytical grade.

4B.2.2. Assay for α-galactosidase activity

The enzyme assay and its quantification was carried out as described for α-mannosidase [Chapter 4A, Section. 2.2] except that the substrate used here was *p*-nitrophenyl-α-D-galactopyranoside. The activity of the enzyme (units/ml/min) was calculated according to the formula given below: (Enzyme activity is measured as described in chapter 4A).

4B.2.3. Extraction and purification of α-galactosidase from *Moringa oleifera*

All the purification steps were carried out at 4°C. The protein elution profile from different chromatographic columns was monitored my absorbance at A_{280}.

50 g of defatted *Moringa oleifera* seed kernels powder was kept for overnight extraction in 25 mM NaOAc buffer pH 6.0 containing 150 mM NaCl (buffer A). After extraction the sample was centrifuged (12,000 rpm for 30 min). The protein in clear supernatant (crude enzyme) was precipitated using 0-80% ammonium sulfate. The precipitate was recovered by centrifugation and dialysis against 25 mM NaOAc buffer pH 6.0 (buffer B).

Cation exchange chromatography using CM-Cellulose

Following dialysis the sample is again centrifuged to remove insoluble materials and the clear supernatant was loaded onto a Cation exchanger CM-Sepharose column (2.5 cm x 6 cm) which is previously equilibrated with 25 mM NaOAc buffer pH 6.0 (buffer B). After washing the unadsorbed proteins with the same equilibrating buffer, the bound protein was eluted with a stepwise elution with NaCl in buffer B starting from100 mM, 150 mM and 300 mM at the flow rate of 40 mL/h. 2mL fractions were collected and checked for both A_{280} and α-galactosidase activity. The fractions containing α-galactosidase activity were pooled and solid ammonium sulphate was added to 1 M concentration.

Hydrophobic interaction chromatography using Phenylsepharose CL-4B

The sample obtained above was loaded onto a phenyl-Sepharose CL -4B column (1 cm x 10 cm) which is previously equilibrated with 25 mM NaOAc buffer pH 6.0 (buffer B) containing 1 M ammonium sulphate. Unbound protein was washed with equilibration buffer and the bound proteins were eluted in absence of ammonium sulphate at the flow rate of 1 mL/min.

Gel exclusion chromatography using Sephadex G-150

The enzyme rich fractions from Phenylsepharose was concentrated using amicon concentrator and applied on to the Sephadex G-150 column (2 cm x 125 cm) previously equilibrated with 25 mM NaOAc buffer pH 6.0 containing 150 mM NaCl (buffer A). The flow rate was 15 mL/h, and the protein in the eluates was monitored at 280 nm.

4B.2.4. Native molecular mass determination

Sephadex G-150 (2 x 125 cm) was equilibrated with 25 mM sodium acetate buffer pH 6.0 containing 150 mM NaCl and 0.02% (w/v) sodium azide. The column was precalibrated using known protein standards: Phosphorylase (96 kDa), BSA (66 kDa), Ovalbumin (45 kDa), and Lysozyme (14 kDa). The apparent molecular mass of the purified α-galactosidase was estimated by passing the enzyme on this gel. The proteins were eluted with same buffer at a flow rate of 15 mL/h. The eluted protein concentration was determined by measuring the absorbance at 280 nm and the enzyme activity was monitored by standard enzyme assay.

4B.2.5. Protein and carbohydrate estimation

Protein concentrations were determined according to Bradford [1976] using BSA as standard. The total neutral sugar content of the enzyme was determined by phenol-sulphuric acid method of Dubois *et al.,* [1956] using glucose as standard. Absorbance at 280 nm was also used to estimate protein concentration in chromatographic fractions.

4B.2.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out in accordance with the procedure of Laemmli [1970], using a 12% resolving gel and a 5% stacking gel under both reducing and nonreducing conditions. The samples were

cooked at 95°C for 5 min with sample buffer (4 X) mixed in 3:1 ratio. The gel was stained with Coomassie brilliant blue. The proteins are compared with Fermentas unstained protein markers as standards: (116.0 kDa) β-galactosidase, (66.2 kDa) Bovine serum albumin, (45.0 kDa) Ovalbumin, (35.0 kDa) Lactate dehydrogenase, (25.0 kDa) REase Bsp98I, (18.4 kDa) β-lactoglobulin, (14.4 kDa) Lysozyme.

4B.2.7. Optima pH and pH stability

The effect of pH on the galactosidase activity was determined by carrying enzyme assay in 100 mM of various pH buffers ranging from pH 2.0 to 9.0. The buffers used were Glycine-HCl buffer (pH 2-3), NaOAc buffer (pH 4-5), Sodium phosphate buffer (pH 6-7), Glycine-NaOH (pH 8-9). The enzyme activity was carried out at 37^{o}C. The pH stability was also carried using the same buffers where the enzyme is pre-incubated for 24 h at 37^{o}C and the residual activity of the liquated enzyme was assayed using standard enzyme assay.

4B.2.8. Temperature optima and stability

Determination of optimum temperature for the α-galactosidase was performed with *p*-nitrophenyl-α-D-galactopyranoside (5 mM) in 100 mM NaOAc pH 5.0 using incubation temperatures in the range of 30 to 80°C. Thermal stability was investigated after incubation of the enzyme at again 30 to 80°C for 1 h an aliquot was drawn and immediately cooled. The residual enzyme activity was determined by standard assay method. The activity of the untreated enzyme was used as the control (100%).

4B.2.9. Effect of various metal ions, sugars and inhibitor reagents

The effect of various metal ions, simple sugars, reducing agents and inhibitor reagents on the enzyme activity was tested by the standard assay with enzyme samples pre-incubated with each of the compounds tested for 30 min at 37°C.

4B.2.10. Substrate specificity and kinetic studies

The relative substrate specificity of α-galactosidase towards various synthetic glycosides was determined at 5.0 mM of substrate concentrations. The Michaelis–Menton kinetic parameters (K_M and V_{max}) were determined by incubating the enzyme at optimum temperature/pH with different concentrations of substrate.

4B.2.11. Chemical modification studies

Chemical modification studies of the purified α-galactosidase were done using NBS in acetate buffer (20 mM, pH 5.0), PMSF in methanol and DEPC in phosphate buffer (20 mM, pH 6.0) which are, tryptophan, serine and histidine modifying agent, respectively. Purified enzyme 0.5 mL was incubated with 0.5 mL modifying agent (20

mM), so as to get a final concentration of the modifying agent as 10 mM, for 30 min, followed by estimation of residual enzyme activity.

4B.2.12. Isolation of protein bodies from the seeds of *M.oleifera*

M.oleifera seeds were soaked overnight, placed on a wet filter paper. After soaking the seed coat is separated from the kernel. The fresh kernel was then chopped with a razor blade into small pieces. The sliced kernel pieces were homogenized using a mortar and pestle in 20 mM HEPES buffer pH 7.4, containing 250 mM Sucrose, 2 mM $MgCl_2$, 1 mM EGTA and 1 mM EDTA. The homogenate was then filtered through a fine cloth. The cell walls and debris were removed by centrifugation at 1,000 x g for 5 min and the supernatants used for the isolation of protein bodies. The supernatants were layered onto a sucrose density gradient solution from 10-65% sucrose as shown in the figure [4B.6-A] and centrifuged at 60,000 x g for 2 h. Distinct bands formed after sucrose density gradient centrifugation were carefully aspirated and visualized microscopically. The suspension containing the protein bodies was washed with 20 mM HEPES buffer pH 7.4, sonicated and centrifuged. The supernatant was analyzed for the α-galactosidase activity and proteins identified by 12% SDS-PAGE.

4B.3.0. Results

4B.3.1. *M.oleifera* α-galactosidase purification

The defatted *M.oleifera* seed kernel when extracted in the acidic buffer like sodium acetate pH 6.0 and when analyzed for the glycosidase activities contained significant amount of α-galactosidase addition to other glycosidases.

In CM-cellulose column, almost all the enzyme activity was retained on the column. The elution pattern with NaCl showed two peaks. The first peak eluted in 100 mM NaCl was devoid of α-galactosidase activity. The second peak eluted with 150 mM NaCl contained the enzyme [Figure: 4B.1-A]. The pooled and concentrated eluate from CM-cellulose was applied on phenyl Sepharose gel at high ammonium sulphate concentration. The enzyme specifically bound on this hydrophobic gel and could be easily desorbed using buffer without salt [Figure: 4B.1-B]. To further separate the enzyme from other contaminating proteins and to determine its molecular mass the eluates were pooled, concentrated and were subjected to gel filtration on G-150. The protein was eluted as a single peak with apparent molecular mass was 66 kDa [Figure: 4B.2 A & B]. α-galactosidase was purified from *M.oleifera* seeds using a combination of several chromatographic steps, the results were summarized in Table: 4B.1. Around 29.6 fold purification was obtained with total yield of approximately 10.5%.

When this sample was analysed using SDS-PAGE it gave a single protein band at approximately 66 kDa, under both reducing and non-reducing conditions confirming that the isolated α-galactosidase is a monomeric enzyme consists of single polypeptide chain [Figure: 4B.3]. The purified enzyme did not contain any covalently bound carbohydrate as evidenced by phenol-sulphuric acid method.

4B.3.2. Biochemical characterization of *M.oleifera* α-galactosidase

The optimum pH of *M.oleifera* α-galactosidase activity was determined to be around pH 5.0 at 37°C, the enzyme exhibited maximum activity (more than 70%) in the pH range of 4.0 to 6.0, only 43% and 10% of the activity remained at pH 7.0 and 8.0 respectively. The pH optimum after incubating the enzyme in different pH buffers indicates that the enzyme was more stable in the acidic environment and lost its activity gradually as the pH increases to the basic side. Only 24% of the activity was retained at pH 8.0 after incubating for 24 h [Figure: 4B. 4-A]. The activity is irreversibly lost at neutral pH. The effect of temperature was studied in the range of

30-80°C, α-galactosidase activity increases with temperature up to 50°C after which it decreased. On reaching 80°C only 10% of the activity remained [Figure: 4B.4-B]. The thermostability of the enzyme was determined by preincubating the enzyme at various temperatures ranging from 30 to 80°C for 30 min. More than 80% of the activity was seen at 50°C after 1 h of incubation.

The effect of various chemical agents and metal ions was tested against the *M.oleifera* α-galactosidase activity is shown in Table: 4B.2. Of all the divalent metals tested Hg^{2+}, Ag^{2+}, Cu^{2+} and denaturant like SDS showed potent α-galactosidase inhibition at the concentration of 1 mM. On the contrary other metal ions such as Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+} and Ni^{2+} didn't show any significant reduction of enzyme activity at 1 mM concentration. Among various sugars tested galactose showed 60% inhibition at 10 mM concentration. Guanidine hydrochloride did not show any effect and urea slightly enhanced the activity possibly due to the exposure of the catalytic site of the enzyme to the substrate. The enzyme did not exhibit any hemagglutinating property.

4B.3.3. Substrate specificity and kinetic studies

Results of the substrate specificity towards synthetic substrates are summarized in Table: 4B.3. The K_m and V_{max} value was measured using *p*-nitrophenyl-α-D-galactopyranoside and was found to be 0.49 mM and 0.88 U/mg respectively.

4B.3.4. Chemical modification studies

Chemical modification studies were carried out to get information about amino acid residues involved in the active site of the enzyme. The involvement of tryptophan and histidine in the catalytic activity is evident by the modification of these residues, which resulted in significant loss of activity by NBS and DEPC treatment. Around 94% and 68% decrease in the enzyme activity was observed with NBS and DEPC treatment, respectively. No loss of activity was observed in presence of PMSF [Figure: 4B.5]

4B.3.5. Detection of α-galactosidase activity in protein bodies

In order to identify the localization of this acidic hydrolase we purified, we isolated the protein bodies from the seeds of *M.oleifera* by sucrose density gradient centrifugation as described under methods [Figure: 4B.6-A]. Several band regions were identified after the centrifugation and the band region corresponding to the 45% sucrose contained largely intact protein bodies when observed microscopically

[Figure: 4B.6-B]. These were further processed to analyze the enzyme activity and the protein composition. The α-galactosidase activity was mainly concentrated in the soluble fraction of the protein bodies together with other storage proteins [Figure: 4B.6-C].

FIGURES

Figure: 4B.1 A & B

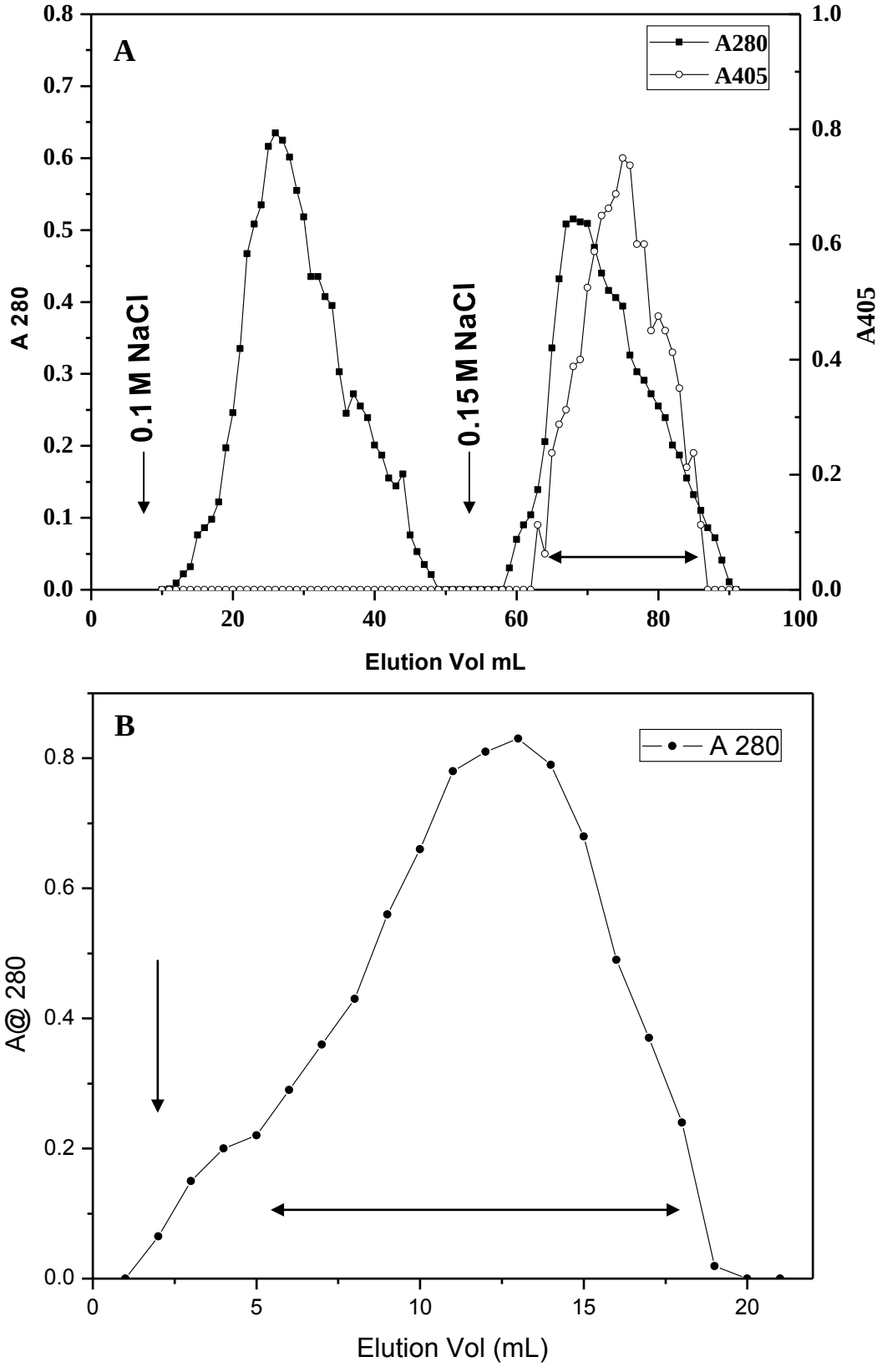
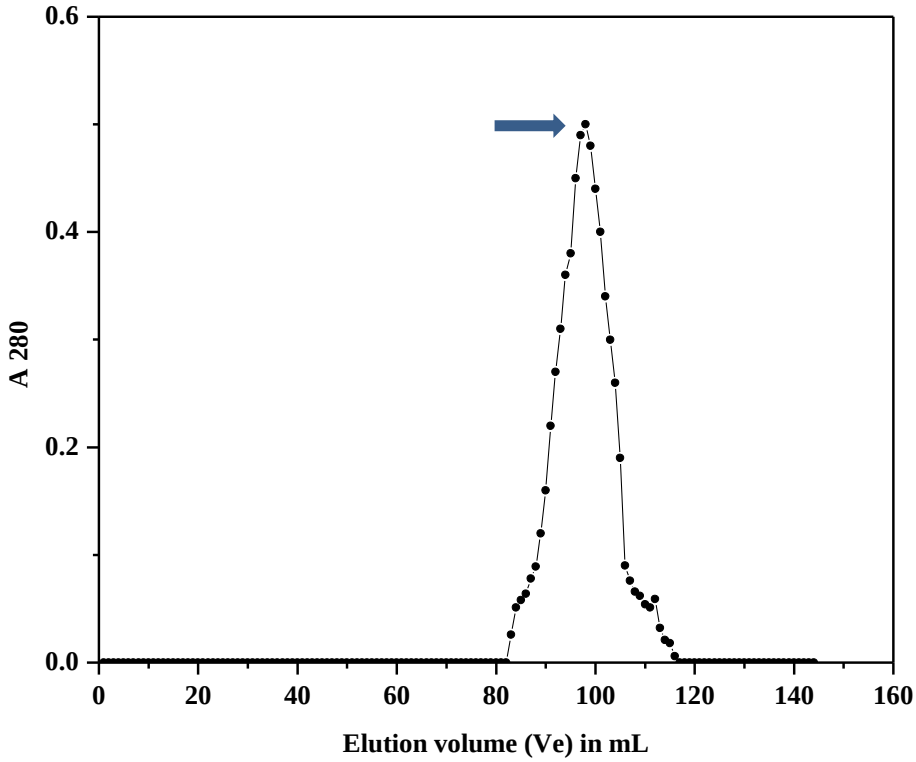
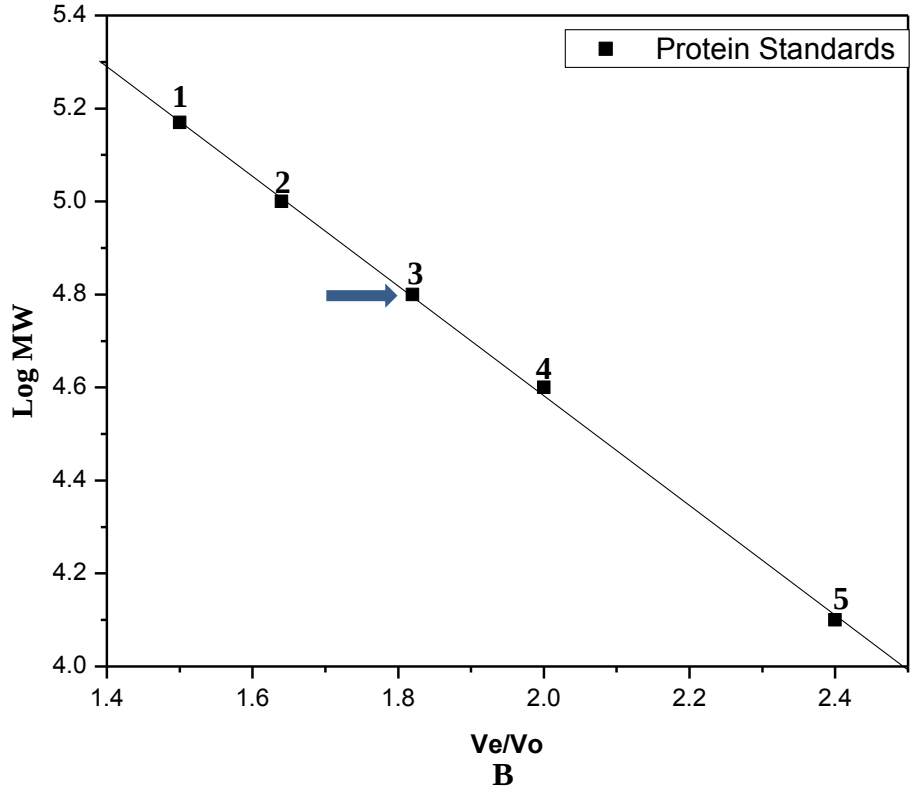


Figure: 4B. 1 A & B. (A) CM-cellulose chromatography: The ammonium sulphate precipitated proteins from the crude extract after dialysis against 25 mM NaOAc buffer pH 6.0 was applied on to CM-cellulose gel (2.5 x 6 cm) pre equilibrated with 25 mM NaOAc buffer pH 6.0. After washing extensively with the equilibration buffer, elution was carried out with one bed volume of 100 mM NaCl followed by 150 mM NaCl. Arrows indicates point of application of NaCl. Bar indicates the enzyme active fractions pooled **(B) Elution profile of *Moringa oleifera* α-galactosidase from phenyl Sepharose:** Enzyme rich fractions from CM–Cellulose chromatography were concentrated and made to 1M ammonium sulphate and applied on a phenyl Sepharose gel (1 x 10 cm) equilibrated with 25 mM NaOAc buffer pH 6.0 containing 1 M ammonium sulphate. After washing the gel with this buffer, bound protein was eluted using 25 mM NaOAc buffer pH 6.0, without salt. 1 mL fractions were collected and protein monitored at 280 nm and assayed for α-galactosidase activity. Bar indicate enzyme active fractions.

Figure: 4B. 2 A & B.



A



B

Figure: 4B.2 A & B: Gel filtration and Molecular weight determination of the α-galactosidase: (A) The phenyl Sepharose eluates containing α-galactosidase were pooled, concentrated and applied on Sephadex G-150 (2 x 125 cm) gel filtration column equilibrated with 25 mM NaOAc buffer (pH 6.0) containing 0.15 M NaCl . 1.0mL fractions were collected. **(B)** Standard graph of known molecular weight proteins: 1. Alcohol dehydrogenase 150 kDa. 2. β-galactosidase 116 kDa. 3. BSA 66 kDa. 4. Ovalbumin 45 kDa and 5. Lysozyme 14 kDa. Arrow indicates point where α-galactosidase was eluted.

Figure: 4B.3

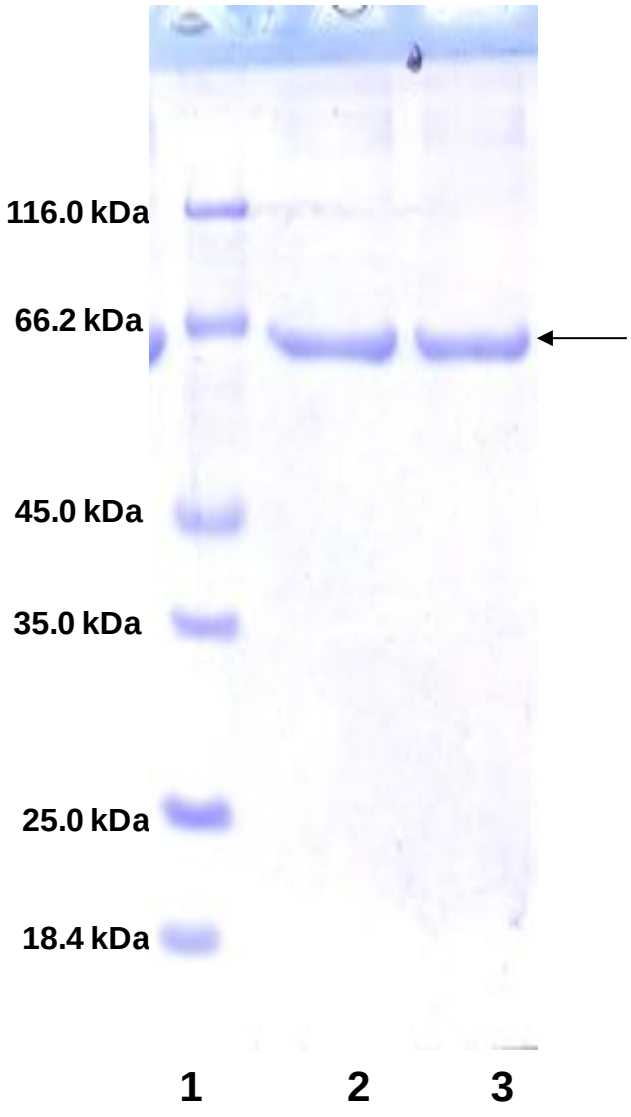


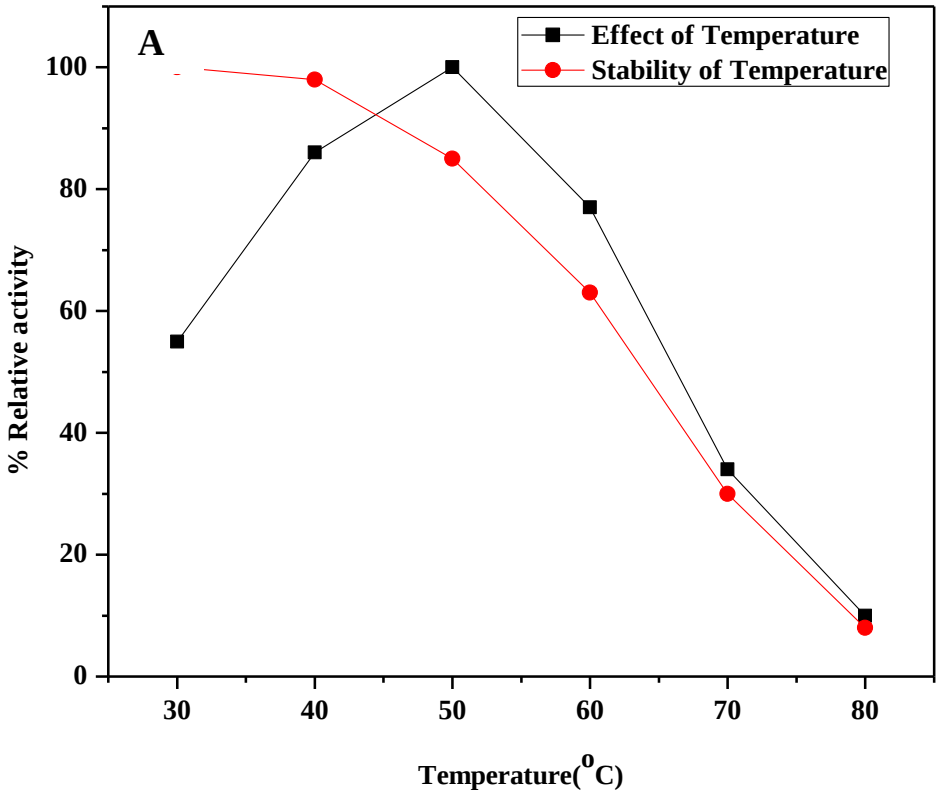
Figure: 4B.3: 12% SDS-PAGE analysis of the purified α-D-galactosidase:

Lane 1. Molecular weight markers (Fermentas),

Lane 2 and 3, Purified α-galactosidase sample after gel filtration under reducing and non-reducing conditions respectively.

Arrows corresponds to the α-galactosidase band**.**

Figure: 4B. 4 A & B



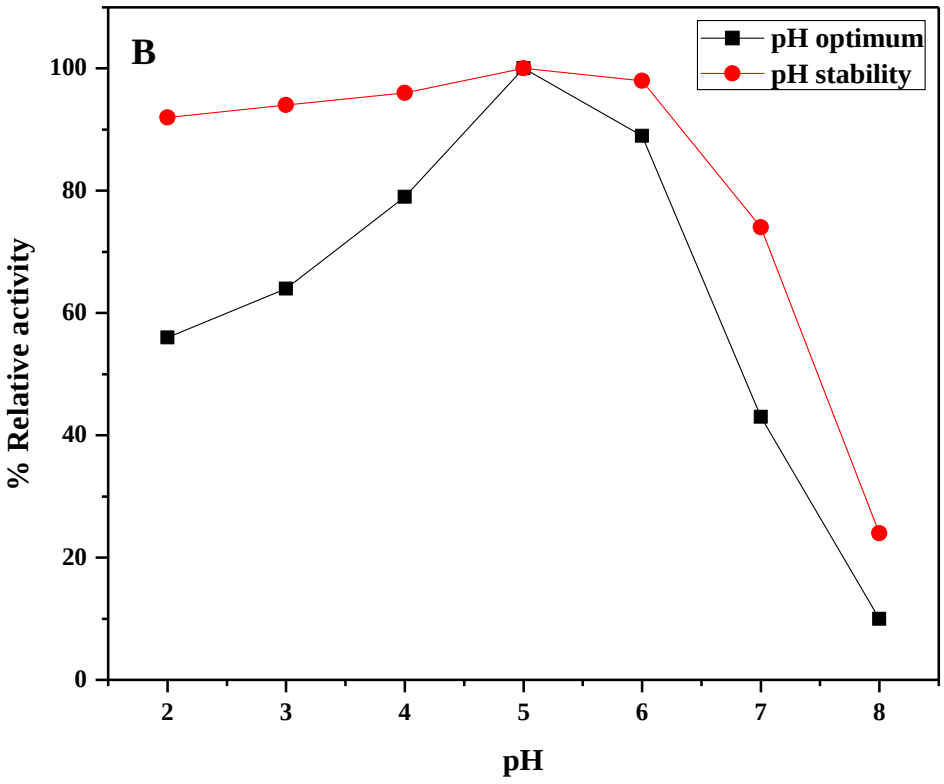


Figure: 4B.4 A & B.

(A) Effect of pH and pH stability: Effect of pH on enzyme activity was carried out by incubating the enzyme with different buffers (pH ranging from pH 2-8) and the pH stability of the enzyme was determined by pre-incubating the enzyme at same pH for 24 h, the residual activity was determined using standard assay. Relative activity was calculated in relation to pH giving highest activity considered as 100%.

(B) Effect of temperature and Temperature stability: The enzyme activity was carried out by incubating the enzyme at different temperatures ranging from 30-80°C. The stability of the enzyme was determined by incubating the enzyme at various temperatures ranging from 30-80°C for 1 h and the residual activity was determined using standard assay. Relative activity was calculated in relation to temperature giving highest activity considered as 100%.

Figure: 4B.5

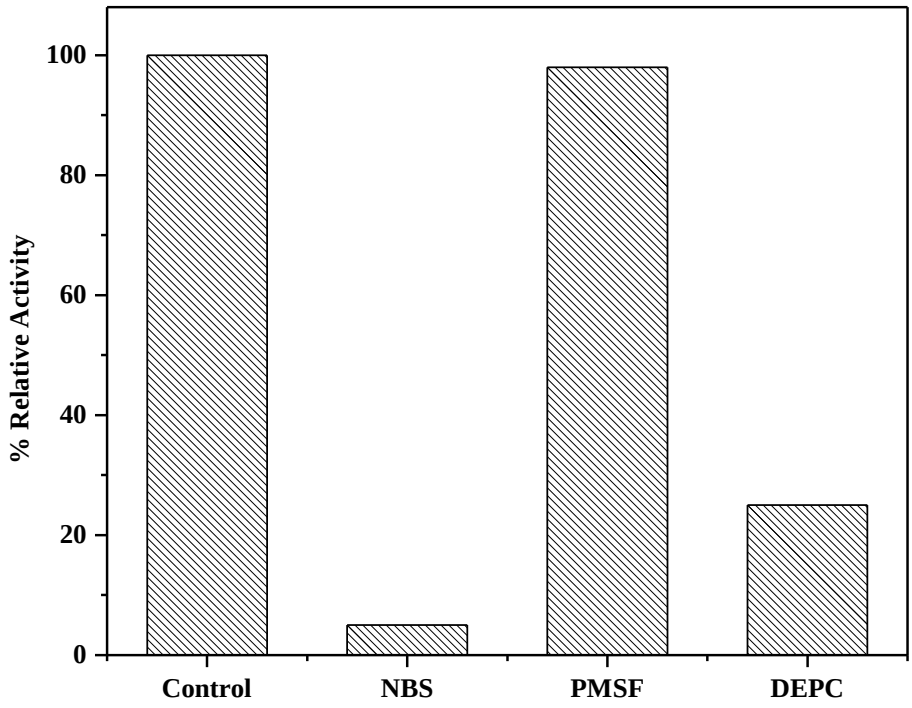


Figure: 4B.5. Chemical modification studies. Purified *Moringa oleifera* α-galactosidase enzyme was incubated with modifying agent, so as to get a final concentration of the modifying agent as 10 mM for 30 min and the residual activity was later assayed using standard assay condition. Sample without modification is taken as 100% (Control).

Figure: 4B.6A, B & C.

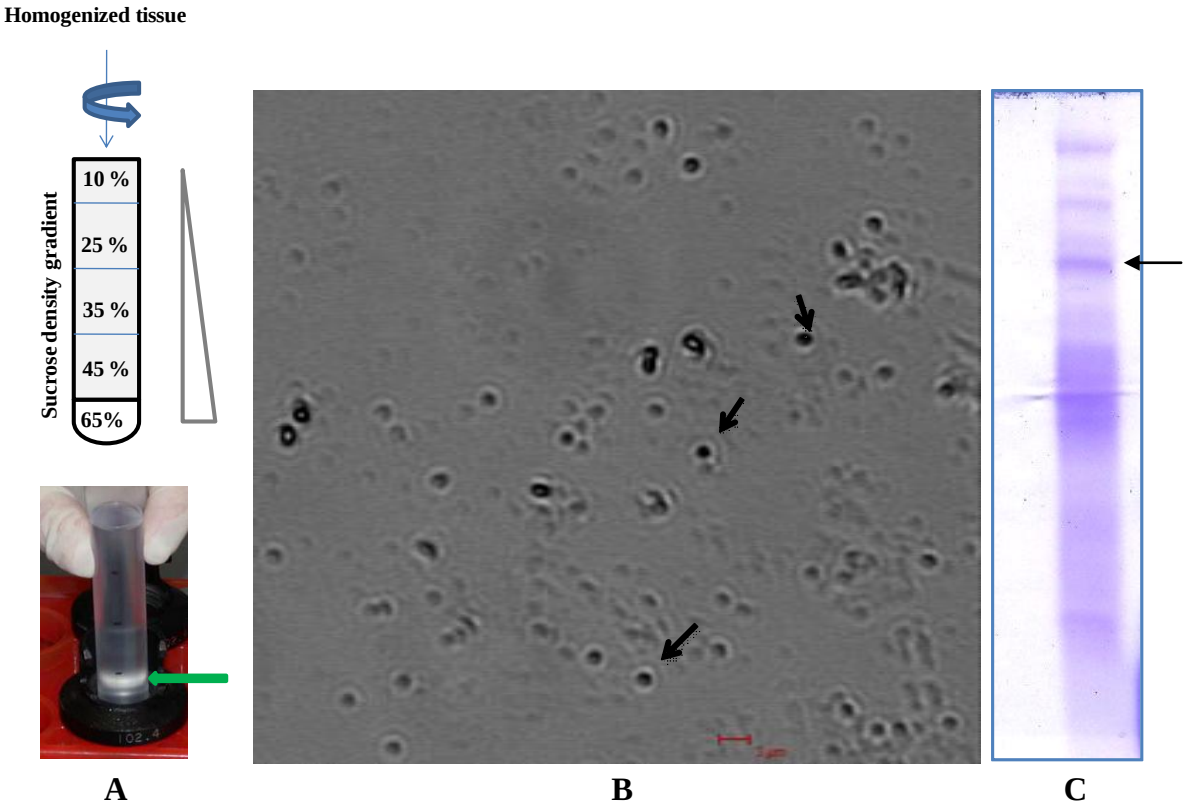


Figure: 4B.6 A, B & C. Isolation of protein bodies: (A) Schematic representation of the sucrose density gradient centrifugation carried out in this experiment, **(B)** Micrograph picture of Protein bodies and **(C)** 12% SDS-PAGE of the proteins extracted from the protein bodies. **(A)** The overnight soaked seed kernel were homogenized in the fractionation buffer, filtered and layered on to the sucrose density gradient. Centrifuged at 60,000 x g for 2 h. Distinct layers formed after centrifugation was carefully aspirated and checked for the enzyme activity. Arrow indicates the layer which has α-D-galactosidase activity. **(B)** The protein bodies isolated from the seed kernel of *Moringa oleifera* using sucrose gradient density centrifugation was visualized using confocal microscope transmission image. Arrow indicates protein bodies. **(C)** The proteins from the protein bodies were extracted by sonicating and thus extracted protein were passed through CM-cellulose column and the bound protein was eluted with 150 mM NaCl. The eluted protein was analyzed using 12 % SDS-PAGE. Arrow indicating the possible α-galactosidase protein.

TABLES

Table: 4B.1. Purification of α-galactosidase from *Moringa oleifera* [a]

Purification stage	Total protein (mg)	Total Activity (units)[b]	Specific Activity [c]	Purification fold	Yield
Crude extract [a]	1400	840	0.6	1	100
CM-cellulose	260	423	1.6	2.6	50.3
Phenyl Sepharose CL-4B	20	136	6.8	11.3	16.1
Sephadex G-150	5	89	17.8	29.6	10.5

[a] 50 g of defatted *Moringa oleifera* seed kernel powder was used for the purification in different chromatographic steps. Proteins were measured in chromatographic fractions at 280 nm.

[b] One unit of enzyme activity was defined as amount of enzyme hydrolyzing 1 µmol of *p*-nitrophenol from *p*-nitrophenyl-α-D-galactopyranoside per min at pH 5.0 and 37°C.

[c] The specific activity of the enzyme is expressed as units per mg protein. (U/mg of protein).

Table: 4B. 2. Effect of chemical agents, sugars and divalent metal ions on α-galactosidase activity.

Effector agent [a]	Relative galactosidase activity (%)[b]
Control	100
EDTA	94
β-mercaptoethanol	95
Iodoacetamide	89
D-Galactose	40
D-Mannose	91
D-Glucose	95
SDS	ND
Mg^{2+}	98
Mn^{2+}	94
Cu^{2+}	ND
Ca^{2+}	90
Hg^{2+}	ND
Fe^{2+}	96
Zn^{2+}	89
Fe^{2+}	94
Ag^{2+}	ND

[a] Sugars and EDTA was 10 mM. Concentration of β-mercaptoethanol was 1% (v/v). All the concentrations of the metal ions and chemical reagent was 1mM.

[b] Relative activities were calculated in relation to control without any addition of effector, this was considered as 100%. ND. Not Detected.

Table: 4B. 3. Relative substrate specificity of purified α-galactosidase from *M.oleifera.*

Substrates [a]	**Relative activity (%)** [b]
pNP-α-D-galactopyranoside	100
pNP-β-D-galactopyranoside	12
pNP-α-D-mannopyranoside	2
pNP-α-D-glucopyranoside	20

[a] Enzyme activity was determined using synthetic substrate as described in section 4B.2.2. at 37°C .

[b] The activities are expressed as percentage of the activity calculated with p-nitrophenyl α-D-galactopyranoside which is taken as 100%

4B.4.0. Discussion

We studied some enzymatic properties of α-galactosidase from *M.oleifera.* The molecular weight of the purified enzyme was about 66 kDa as determined by both gel filtration and SDS-PAGE analysis, suggesting a monomeric form. Other plant α-galactosidases, *Vitis vinifera* [Kang and Lee, 2001], *Oryza sativa* [Kima *et al.,* 2002] and *Helianthus annuus* [Kim *et al.,* 2003] have molecular masses in the range 40-50 kDa. The pH optimum of the enzyme was around 5.0 when *p*-nitrophenyl α-D-galactopyranoside (*p*NP-α-Gal) was used as a substrate. The decline in activity of the enzyme beyond pH 6.0 and exhibiting optimal activity at pH 5.0 suggests this enzyme to be acidic nature. Similar findings are reported in other plant galactosidases [Gao and Schaffer, 1999]. Most of the plant α-galactosidases are thermally stable and the *M.oleifera* was also stable. However, α-galactosidases from microbial sources exhibit more thermal stability. Reducing agents like β-mercaptoethanol and iodoacetamide did not affect the enzyme activity significantly suggesting non involvement of sulfhydryl groups in the activity. Absence of inhibition in presence of 10 mM EDTA suggests that this enzyme is not a metalloenzyme. This is in agreement with the other α-galactosidase [Viana *et al.,* 2005]. The effect seen with the SDS could possibly be due to the loss of enzyme conformation as has been reported for other enzymes. The inhibition of α-galactosidase with Hg^{2+} and Cu^{2+} ions is usually attributed to its reaction with thiol, carboxyl, amino and imidazolium groups, whereas inhibition with Ag^{2+} may be attributed to the reaction with only carboxyl and or histidine residues at the active site of α-galactosidase. Most of the metal ions tested for the effect on catalytic activity of α-galactosidase do not appear to have stabilizing/activating effect. Similar effects were seen with the enzymes isolated from other sources such as the *T. multijuga* seeds [Fialho *et al.,* 2008], water melon seeds [Itoh *et al.,* 1986] and soya bean seeds [Viana *et al.,* 2005]. Additionally, it is known that the *Oryza sativa* enzyme also lost its activity in presence of these metal ions and it is suggested that this is due to its binding at the catalytic site preventing the substrate from binding to the enzyme [Li *et al.,* 2007]. The inhibition of α-galactosidases by various sugars and their derivatives has also been reported [Chinen *et al.,* 1981]. Generally D-galactose is a competitive inhibitor of many α-galactosidases [Agnantiari *et al.,* 1991; Gao and Schaffer, 1999], which may be due to the fact that this sugar is a substrate analogue.

α-galactosidase from *Glycine max* and other legume are associated with hemagglutinin properties [Hankins *et al.*, 1980]. Previous study on the lectin form the same source [Tejavath *et al.*, 2011] it was described that the interaction of the glycosidases with that of lectin affigel under acidic conditions. Further, in the initial purification steps of MoSL lectin using galactose column, α-galactosidase was appeared as a minor contaminant, owing to support the concept of MoSL interactions with α-galactosidase from the same source. They might co-localized in the same compartment. Earlier reports on plant α-galactosidase showed that tryptophan, and histidine is important amino acids present at the catalytic site of this enzyme [Zhu *et al.*, 1995; Zhu *et al.*, 1996; Maranville and Zhu, 2000]. To cross check if it is true for the *M.oleifera* α-galactosidase, amino acid specific chemical modification studies were carried out with this enzyme. It was observed the both NBS and DEPC contributed to the loss of enzyme activity suggesting their role of tryptophan and histidine respectively in the enzyme activity.

Though the α-galactosidase has been reported from some plant seeds, little information are known about its specific localization in the seeds. Among the legumes, the plant glycosidases together with the seed storage proteins and lectins have been shown to be present in protein bodies of the seeds suggesting possible *in vivo* interactions among these different proteins that may be of physiological significance during seed growth and development.

The specific localization of the enzyme to the protein bodies strongly supports the possible *in vivo* function of this enzyme during the seed growth and development in removing galactose residues from protein/oligosaccharides. The α-galactosidase synthesized during the seed development is possibly targeted to protein bodies. Natural substrates for this enzyme such as the raffinose oligosaccharides, and galactosyl cyclitols accumulate mainly in the cytosol. During germination, there is considerable swelling of the protein bodies to form a vacuole, where in the enzyme can preferentially act on the natural substrates and aid in their hydrolysis for effective utilization of the products by the growing seedling [Viana *et al.*, 2005]. In the present study, identification of the protein bodies and the enzyme as one of the constituent proteins of the protein bodies, strongly suggests the *in vivo* function of this enzyme during seed germination.

In conclusion, we have purified α-galactosidase from *M.oleifera*, and studied various properties of the enzyme, which show both similarities and differences from other α-galactosidases reported earlier. Future studies on the enzyme to determine its complete sequence by proteomics approach or by molecular cloning should reveal the relatedness of the *M.oleifera* enzyme to other enzymes.